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Canadian Journal of Research

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VOL. 28, SEC. E. DECEMBER, 1950 NUMBER 5

VIBRIO CHOLERAE IN FLUID MEDIA[1]

BY L. E. RANTA[2] AND MARY MCLEOD[3]

Abstract

Studies have been made of the growth of *V. cholerae* in fluid media of chemically defined compositions. The addition of three amino acids, tyrosine, asparagine, and glycine, to a fluid medium containing inorganic salts produced a growth of *V. cholerae* equivalent to a 450 p.p.m. silica standard. Under conditions of aeration with an air and carbon dioxide mixture, yields comparable to the turbidity of a 1600 p.p.m. silica standard were obtained with a medium composed of 0.67 gm. of tyrosine, 0.42 gm. of asparagine, 0.51 gm. of glycine, 5.0 gm. of sodium chloride, 5.0 gm. of ammonium sulphate, 0.75 gm. of dipotassium hydrogen phosphate, 0.1 gm. of magnesium sulphate, 10.0 gm. of glucose, and 15.0 gm. of sodium bicarbonate dissolved in one liter of distilled water.

As cholera has not invaded this continent since 1873, the demand for cholera vaccine in Canada is ordinarily small. It is required only to satisfy the needs of travellers into cholera endemic areas in the Near and Far East, where the disease still counts its victims in the hundreds of thousands every year. In view of the existence of such a nidus, there is always a possibility that large quantities of cholera vaccine may suddenly be required, even in Canada, to immunize a threatened or invaded population.

The method for the production of cholera vaccine as employed in Canada was reported by Ranta and Dolman (8) in 1943. In this method, selected strains of *V. cholerae* are grown on solid medium and harvested from it with phenolized saline. Production in this way necessitates considerable handling of materials in relatively small batches, and large-scale production becomes cumbersome.

In 1944, Linton and Jennings (5, 2, 3) reported on a procedure for the development of a heavily turbid growth of *V. cholerae* in a buffered casein digest fluid medium, in which the yield had been enhanced by aeration with a carbon dioxide and air mixture. Though Linton and Jennings were primarily interested in a heavy yield for their biochemical studies of the vibrios, it did not

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This report is based upon a paper presented at the Western Regional Meeting of the Medical Section of the National Research Council, Winnipeg, Man., 9 February, 1950. The study was supported by a National Public Health Grant and received the co-operation of the Department of Bacteriology and Preventive Medicine and of the Western Division of Connaught Medical Research Laboratories, University of British Columbia.

[2] *Associate Professor, Department of Bacteriology and Preventive Medicine, University of British Columbia, and Research Associate, Connaught Medical Research Laboratories, University of Toronto.*

[3] *Research Assistant, Department of Bacteriology and Preventive Medicine, University of British Columbia.*

escape them that this method might be adapted for vaccine production. But its value as a means for producing an effective vaccine was apparently not tested.

As inoculation with digested proteins may result in local and, rarely, generalized reactions, safety demanded the use of the simplest medium that would support an abundant growth of *V. cholerae* in a satisfactorily antigenic form. Moreover, any newly developed cholera vaccine must compete with the present vaccine, which is probably the most benign product among the active immunizing agents. These considerations led to an investigation to determine the results of growth of *V. cholerae* in chemically defined fluid media. The work of Koser and Rettger (4) in 1919, and Robertson (10) in 1924, on the nutrition of *V. cholerae*, and the fact that peptone water alone supports ample growth, encouraged the search for a simple synthetic medium.

Experimental Procedures

V. cholerae, Strain No. 41 (Ogawa) was used throughout the study. All media were adjusted to pH 8.5 before sterilization, and to effect rapid and uniformly dispersed growth, instead of a surface pellicle, provision was made for the aerobic utilization of a simple sugar. The mineral demands were satisfied by a basic medium containing 5.0 gm. of sodium chloride, 0.75 gm. of dipotassium hydrogen phosphate, and 0.1 gm. of magnesium sulphate, in one liter of distilled water. When this was put to use, amino acids and glucose were added, the latter in quantities of 0.1, 0.2, and 0.3%. As growth occurred in the basic mineral medium when an enzymatic digest of casein was added, it was decided to incorporate amino acids into the basic medium in the percentages reported for casein. The fallacy of this conclusion was fully appreciated, but the procedure was meant to serve merely as a starting point. Accordingly, appropriate concentrations of 20 amino acids were made up in 50 ml. quantities.

In selecting the amino acids for study, some guidance was also afforded by the work of Mitra (6, 7) in 1936, who reported on the racemization of the proteins of *V. cholerae*. Thus, special attention was paid to the 12 amino acids that he identified: glycine, alanine, valine, leucine, tyrosine, aspartic acid, glutamic acid, proline, hydroxyproline, arginine, histidine, and lysine.

The basic medium, with amino acids added, was dispensed in 5 ml. quantities into large test tubes. During incubation the tubes were slanted as much as practicable in order to facilitate aeration. This medium was seeded with 0.4 ml. of a culture grown in casein digest basic medium, but the effect of the small quantity of casein digest transferred in the inoculum was obliterated by four serial transfers in each amino acid basic medium at 24 hr. intervals. As a control, each series of tests was accompanied by an inoculated tube of casein digest basic medium.

Observations were made at intervals up to 64 hr. after inoculation, and the growth, if any, was compared with silica standards (1), ranging in steps of 50 p.p.m. from 100 to 1000 p.p.m.

At first, each amino acid was tested separately. Then, pairs of amino acids were tried, but it was not necessary to use more than 45 of the 190 possible paired combinations, for omissions were indicated when experiments in this paired series showed a member of a pair to be inhibitory in its action upon *V. cholerae*. For further study, single amino acids were added to pairs that had given the most promising growth. Later, certain pairs of amino acids were similarly added to other pairs, so that the greatest number in any of the tested media did not exceed four amino acids.

When a suitable combination of amino acids was found, *V. cholerae* was grown in liter quantities of the medium after some further mineral salts were added.

During the period of growth these cultures were subjected to continuous aeration. This was carried out by passing air from a compressed air tank through a calibrated glass flowmeter. Before reaching the flowmeter, the gas travelled through the horizontal arm of a T-shaped glass tube, the vertical arm extending downward about one meter. The open end of the vertical arm of the T-tube was immersed in water contained in a tall cylinder, so that the pressure of nearly one meter of water prevented the air from bubbling into the cylinder, but if the gas pressure suddenly surged, the apparatus acted as a satisfactory safety device. A similar appliance served to pass carbon dioxide from a cylinder through a flowmeter. Using a light oil in the U-tubes of the flowmeters, the delivery was adjusted to provide 15 liters of air and 5 liters of carbon dioxide per hour.

The tubes leaving the flowmeters passed the gases through chambers containing sterile cotton wool; then, by a common tube, the gases were led into the incubator and into the depths of a three-necked, 2-liter distilling flask containing one liter of medium. The gases were dispersed into the medium by tying a fine linen bag over the flared end of a glass tube. One of the necks of the flask was fitted with a wide-bored glass tube. This reached just below the surface of the medium. By means of a rubber bulb, the medium could be drawn periodically into the tube in order to compare the turbidity of the growth with silica standard suspensions kept in similar tubes. The third neck was stoppered with cotton wool to permit escape of the aerating gases.

Growth in Nonaerated Amino Acid Basic Media

On the whole, the addition of a single amino acid to the basic medium of mineral salts resulted in sparse growth of *V. cholerae*. After incubation for 24 hr. in the fourth subculture, the best results were obtained with asparagine, the acid amide of aspartic acid. The turbidity equalled that of a 200 p.p.m. silica standard, while casein digest basic medium produced a turbidity of 250 p.p.m. Traces of growth were supported by alanine, valine,

serine, phenylalanine, hydroxyproline, lysine, and arginine; while turbidities up to 100 p.p.m. were obtained with glycine and with isoleucine. Many amino acids supported no growth: *viz.*, histidine, methionine, proline, leucine, tryptophane, cystine, aspartic acid, glutamic acid, and threonine.

Of the pairs of amino acids, tyrosine and glycine produced a density of growth equalling 250 p.p.m., and corresponding to the growth in casein digest basic medium. Tyrosine and isoleucine gave a growth of 200 p.p.m., and tyrosine and alanine developed a turbidity of 175 p.p.m. Only one other amino acid seemed to encourage growth. While the inhibition caused by aspartic acid made it unsuitable, asparagine in combination with tyrosine, histidine, or with glycine produced a growth of either 125 or 150 p.p.m. Thus, the stimulating effect of asparagine, when used alone, was maintained when it was added to some other amino acids. This was not a general rule, for a combination of amino acids with tyrosine fostered the greatest yield, despite its inhibitory action when used alone.

In tests of trios of amino acids, the turbidities obtained upon the addition of an amino acid to combined tyrosine and glycine rarely resulted in an increased yield. Tryptophane and proline both completely inhibited the growth, and several other amino acids, such as, aspartic acid, cystine, hydroxyproline, threonine, methionine, phenylalanine, serine, valine, and histidine, reduced the yield to less than 200 p.p.m. The addition of alanine, isoleucine, or of lysine brought about yields of 300 p.p.m., while arginine increased the density to 375 p.p.m. But asparagine brought about the greatest yield. Instead of 250 p.p.m. as obtained with combined tyrosine and glycine, the addition of asparagine produced a density of 450 p.p.m., or roughly double the yield in casein digest basic medium.

As the yield in tyrosine – asparagine – glycine basic medium (TAG medium) was encouraging, the remainder of this part of the investigation consisted of adding single amino acids to TAG medium. In no case was the yield increased by the addition of an amino acid. In fact, the general response was an appreciable reduction in turbidity to between 125 and 225 p.p.m.

Growth in Aerated Buffered Media

As the results obtained with nonaerated TAG medium gave promise of a reasonably large yield, it was tested by employing aeration and the carbon dioxide/sodium bicarbonate buffering system recommended by Jennings and Linton (3).

For aeration, TAG medium was prepared as follows: 0.67 gm. of tyrosine, 0.51 gm. of glycine, 5.0 gm. of sodium chloride, 5.0 gm. of ammonium sulphate, 0.75 gm. of dipotassium hydrogen phosphate, and 0.1 gm. of magnesium sulphate were dissolved in one liter of distilled water. The pH was adjusted to 8.5 before sterilization in an autoclave at 15 lb. pressure for 20 min. Before inoculation, the following substances were added: 0.42 gm. of asparagine, autoclaved separately in dry form; 10.0 gm. of glucose, autoclaved

separately in a minimum of distilled water; and 15.0 gm. of sodium bicarbonate autoclaved in dry form. Carbon dioxide from a pressure tank was bubbled through the medium for a few minutes before it was inoculated with 100 ml. of an overnight culture of *V. cholerae* in unaerated TAG medium.

The yields after 48 hr. growth in buffered, aerated TAG medium have consistently been over 650 p.p.m., with the majority ranging over 1000 p.p.m., and reaching as high as 1600 p.p.m.

Studies to modify the amino acid concentrations of TAG medium have shown that no material changes are obtained by doubling the concentration of any one, or all of the amino acid constituents, although increases in asparagine reduced the density of growth by approximately one-half. The substitution of the amino acids in TAG medium has shown that comparable yields can be obtained by replacing the asparagine and glycine with 0.97 gm. of isoleucine, the tyrosine with 0.16 gm. of alanine, or the asparagine with 0.39 gm. of arginine.

Discussion

The greatest yields obtained with TAG medium were equivalent to a 1600 p.p.m. silica standard. This density is about thrice greater than the density of cholera vaccine now employed for human immunization. Samples of these and other experimental lots have been preserved by diluting with physiological saline to produce a final concentration of 8000 million *V. cholerae* per ml. The organisms in these suspensions have been killed by the addition of phenol to a final concentration of 0.5%. The vaccines, so prepared, are at present being compared antigenically with the standard type of cholera vaccine, employing the mouse protection test reported by Ranta and Dolman (9) in 1944.

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PARALLEL STUDIES OF COMPLEMENT AND COAGULATION[1]

I. THE EFFECT OF VITAMIN C

By Christine E. Rice and Paul Boulanger

Abstract

Groups of guinea pigs on a basic diet of commercial rabbit pellets supplemented by fresh green grass or stored roots exhibited marked differences in the ascorbic acid level of their plasma. These differences were not accompanied by any significant variation either in the complement titer of the serum or the prothrombin time of the plasma. Such minor variations as were observed in these two activities did not parallel each other.

Introduction

The question of whether the two important normal properties of the blood, complement and coagulative activity, are basically related has interested physiologists and immunologists for many years (4, 6–10, 15–18, 27, 28). Aside from the fact that both phenomena involve the precipitation of blood proteins, the major evidence suggesting the possible relationship of the two reactions has been that they are affected by many of the same factors *in vitro*, while *in vivo* a decrease in both activities has been observed in certain pathological conditions, notably in liver disease and in anaphylactic shock. On the other hand, data indicating that the relative amounts of the various reagents required to inhibit the two reactions may vary widely, and the further observation that the coagulation time of the blood may increase without an accompanying decrease in complement titer, has suggested to other groups of investigators that the two reactions are independent of each other.

Both complement activity and coagulation are, however, complex phenomena involving a series of components, all of which are essential for the completion of the respective reactions. The complement complex consists of at least four major components, two of which are globulins, one apparently a carbohydrate, and one of unknown nature. The coagulation system requires in addition to the earlier-recognized components—prothrombin (thrombin), thromboplastin, fibrinogen (fibrin), and calcium—certain more recently described labile factors (20, 22, 29). Of the elements making up the two systems, it has been claimed that prothrombin and complement midpiece (C′1) are the same entity (6, 7), or at least that the same molecule is involved, with different grouping being concerned in the two reactions (21). Furthermore, the procedures used to inactivate the third and fourth components of complement has been shown to remove thrombin-forming ability as well (18). The well known fact that plasma and serum of the same blood specimen have approximately the same hemolytic complement titer does not necessarily show that coagulation and complement activity are independent phenomena,

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Contribution from the Division of Animal Pathology, Science Service, Dominion Department of Agriculture, Animal Diseases Research Institute, Hull, Que.

since the presence of prothrombin and other elements of the coagulation system may be demonstrated in serum. It does indicate, however, that even if the same or some of the same components are involved, the quantitative relationships between them vary considerably in the two reactions. So much additional information has been accumulated during the past 10 years in regard to both activities that it now seems an opportune time to reinvestigate the problem of their relationship in the light of this recent knowledge.

Thus, when a study of the effect of the vitamin content of the diet upon the complement titer of guinea pig serum was undertaken last spring in connection with another project, it seemed worthwhile to carry out a parallel investigation of the coagulative properties of the plasma and thereby obtain data on both problems simultaneously. The effect of vitamin C nutrition on the two activities will be considered in the present paper.

Literature Review

Following a number of scattered reports on the effect of scurvy on the complement titer of human and guinea pig blood, Ecker and his co-workers (3, 5) demonstrated experimentally that a correlation existed between complement activity and the ascorbic acid level of the blood of guinea pigs. They observed that complement titers rose as the ascorbic acid content of the blood was increased to 1.0 mgm.%, but that above this level, no further increase occurred. Agnew, Spink, and Mickelsen (1, 24), on the other hand, were unable to note any relationship either *in vivo* or *in vitro*, between the ascorbic acid content of human or guinea pig blood and complement titer. Kapnick and Cope (11) recorded a series of experiments on the relationship of basal metabolic rate, complement titer, and the vitamin C intake of rabbits. After thyroidectomy, they found that the complement value of the serum fell whereas its ascorbic acid level remained normal. Conversely when hyperthyroidism was produced in the rabbits by the administration of thyroxin, the complement titer rose as the metabolic rate increased and the vitamin C content of the serum fell. Kodicek and Traub (12) tested groups of guinea pigs on diets supplemented with 0.5, 1.0, and 10 mgm. of ascorbic acid. Using the point of 50% hemolysis as the unit of comparison in their titrations, they found that, although the animals on the vitamin-C-deficient diet had lost weight, their complement titers were not altered significantly. Experiments made in the summer and late fall showed essentially similar results.

Considerable controversy has also arisen as to whether vitamin C affects the coagulability of blood. According to Macrae (14), Sir Almoth Wright described scurvy as "an acid intoxication which eventuates in a defect of blood coagulability," although Macrae himself did not note any appreciable deviation from normal in such persons. Several groups of workers subsequently reported that animals on diets low in vitamin C show an increase in plasma prothrombin time, but in general their conclusions were based on observations with small groups of animals. In a recent study, Sullivan *et al.* (25) found that scorbutic animals showed an increased response to dicumarol which became

greater the longer the deficiency lasted. Prothrombin levels took longer to return to normal after dicumarol injection in guinea pigs fed a vitamin-C-deficient diet than in animals on adequate diets. The observation of Richards and Cortell (23) that, in scorbutic guinea pigs, dicumarol caused fatty infiltration and degeneration of the liver is of interest in view of the important role of the liver in protein metabolism. Indeed, in evaluating the effects of vitamin C on either complement or coagulation, its indirect influence on protein intake due to tooth injury and attendant difficulties in eating must always be taken into consideration.

Methods

Diet of Animals

Guinea pigs were divided on the basis of weight into two groups of 36 each, both of which were fed the same basic diet of commercial rabbit pellets.* The first group was fed fresh green grass as a source of vitamin C and other factors; the second group received a supplement of mangels that had been stored in pits during the winter. The grass collected during the hot weather in July was dry and brittle and the roots at this time had become soft and of poor quality. Thus the vitamin C content of both kinds of supplement was lower than it had been in May.

The animals were weighed periodically. Although the first group showed a greater gain in weight, the second remained in moderately good condition, with only the occasional animal showing mild symptoms of scurvy.

Collection of Blood

Animals from each group were bled from the heart at periodic intervals. Part of the blood was oxalated, the remainder was allowed to clot, and the serum drawn off within two hours.

Coagulation Tests

The oxalated bloods were centrifuged immediately and the plasmas tested for prothrombin activity by the methods of Quick and of Howell (13).

Complement Titrations

The amount of each complement required for 50% hemolysis of a maximally sensitized sheep-red-cell suspension (the unit) was determined as described in the Standard Methods of the Division of Laboratories and Research of the New York State Department of Health (26). Complement titrations were made at approximately 2 and 24 hours after collection of the blood. A small number of sera were titrated for their content in the four major complement components, C′1, C′2, C′3, and C′4, using the method of Bier *et al.* (2).

* *Master Rabbit Pellets from the Toronto Elevator Company, Limited, Toronto, guaranteed to contain a minimum of 15% crude protein, 4% crude fat, and a maximum of 11% crude fiber. The specific ingredients were stated to be as follows:—*

dehydrated alfalfa meal, ground yellow corn and/or hominy feed, alfalfa meal, soybean oil meal, linseed oilcake meal, feeding bone meal, ground wheat, wheat shorts, ground oats, wheat germ, wheat bran, ground limestone, 0.25% iodized salt, manganese sulphate, and fish oil fortified with vitamins A and D.

Ascorbic Acid Determinations

The method of Mindlin and Butler (19) as described for use with the Klett–Summerson photoelectric colorimeter was employed.

Results

In an experiment conducted just previous to the beginning of the present study, it had been found that the majority of guinea pigs on the basic pellet diet, without roots or vitamin C supplement, would develop symptoms of scurvy and a considerable number die without showing any significant decrease in complement titer. A few animals, however, which had become greatly emaciated, had ceased to eat, and were on the point of death, showed complement titers significantly lower than those of guinea pigs on the same basic diet with roots supplement. Aside from the effect of scurvy in reducing the food intake of the animals, the omission of vitamin C from the diet for two to four weeks had no consistent reducing effect on complement activity.

It seemed possible, however, that a state of chronic vitamin C deficiency in which the metabolic disturbance extended over a longer period might be more likely to be reflected in an altered character of the blood constituents. To test the effect of chronic scurvy on complement titer in guinea pigs, two diets were selected, one to supply a large amount of vitamin C (fresh green grass supplement), the other a relatively smaller but sufficient amount to maintain the animals without any marked scorbutic symptoms (stored roots supplement). The experiment was continued from the end of April to about the middle of July.

Ascorbic Acid Values of Plasma

Throughout the period of observation the second group showed very little ascorbic acid in their plasma, whereas in May and early June the first group had high values. In mid-June, after a few days of very warm weather, a decline was noted in the vitamin C level of the plasmas of the first group, a decline traceable in part at least to the decrease in the ascorbic acid content of the grass supplement. The ascorbic acid values for the May bleedings showed considerable irregularity, later found to be due to the presence of traces of copper or other substances in the distilled water diluent. These difficulties were avoided by the use of water redistilled in glass. In view of the inconsistencies in the earlier results they have not been included in Table I.

Complement Titers

Table II, in which the complement titers of the various bleedings are summarized, indicates that the range and mean values for the two dietary groups were closely comparable. Both the 2- and 24-hr. complement titers have been given since it is well known that some complements, initially of very high titer, may deteriorate appreciably on standing overnight in the refrigerator. Such increased lability has been noted more particularly with complements

TABLE I

A COMPARISON OF THE ASCORBIC ACID CONTENT OF THE PLASMA OF GROUPS OF GUINEA PIGS ON DIETS OF COMMERCIAL RABBIT PELLETS SUPPLEMENTED WITH FRESH GREEN GRASS OR STORED ROOTS

Date of bleeding, 1949	Number of guinea pigs	Dietary supplement	Plasma ascorbic acid		
			Range, mgm. %	Mean, mgm. %	Standard deviation
June 1	6	Grass	1.92 – 2.83	2.29	
June 2	8	Grass	1.09 – 1.56	1.30	
June 14	7	Grass	0.32 – 1.72	1.25	
June 15	4	Grass	0.09 – 1.36	0.77	
June 20	4	Grass	0.18 – 2.00	0.74	
July 19	5	Grass	0.36 – 0.90	0.64	
July 25	7	Grass	0.36 – 0.70	0.62	
June 1	5	Roots	0.00 – 0.03	0.01	
June 2	8	Roots	0.00 – 0.18	0.04	
June 15	4	Roots	0.00 – 0.32	0.10	
June 20	8	Roots	0.00 – 0.27	0.12	
July 19	9	Roots	0.00 – 0.07	0.05	
July 25	6	Roots	0.00 – 0.10	0.04	
Mean values	41	Grass	0.09 – 2.83	1.14	0.641
" "	40	Roots	0.00 – 0.32	0.06	0.077

TABLE II

COMPLEMENT TITERS* OF SERA OF GUINEA PIGS ON THE TWO DIFFERENT DIETS

Date of bleeding, 1949	Number bled	Supplement	First day†		Second day†	
			Range, units/ml.	Mean, units/ml.	Range, units/ml.	Mean, units/ml.
May 2	4	Grass	2000 – 2330	2130	1430 – 1820	1560
May 5	3	Grass	1790 – 2080	1960	1540 – 2380	1750
June 1 and 2	14	Grass	1370 – 2500	1790	1150 – 2000	1490
June 14 and 15	8	Grass	1430 – 2780	1890	1220 – 2000	1430
June 20	4	Grass	1920 – 3300	2500	1250 – 1430	1370
July 7	4	Grass	1300 – 1670	1490	1250 – 1820	1540
July 19	5	Grass	1590 – 2200	1720	1370 – 1540	1430
May 2	16	Roots	1560 – 2570	2270	1180 – 1540	1350
May 5	8	Roots	1430 – 2500	1990	1490 – 2280	1840
June 1 and 2	13	Roots	1430 – 2500	1790	1110 – 2220	1410
June 14 and 15	4	Roots	1320 – 2500	1850	1390 – 2330	1610
June 20	15	Roots	1320 – 3300	2330	1370 – 2270	1610
July 7	4	Roots	1250 – 2940	1850	1330 – 2000	1590
July 19	9	Roots	1540 – 2270	1790	1430 – 1820	1590
Mean values	42	Grass	1330 – 3300	1850	1150 – 2380	1490
" "	69	Roots	1250 – 3300	2000	1110 – 2330	1540

* *The titers are expressed in terms of the estimated number of 50% hemolytic units per milliliter of serum.*

† *Tested approximately 2 and 24 hr. after bleeding.*

collected in late winter or after periods of very warm weather. No differences in relative stability were noted, however, in the bleedings from the two groups of guinea pigs.

Ten sera from each group, May bleedings, were titrated for their content in C'1, C'2, C'3, and C'4. No significant differences in mean values were recorded:

Component	Unit of comparison	Dietary supplement, units/ml.	
		Grass	Roots
C'	50% hemolysis	1527	1715
	100% hemolysis	778	926
C'1	100% hemolysis	2330	2105
C'2	100% hemolysis	723	744
C'3	100% hemolysis	1130	1367
C'4	100% hemolysis	6500	4434

Prothrombin Time

The prothrombin time values of the plasmas from the same bleedings are given in Table III. The mean values for the two groups, as determined by the methods of Quick and of Howell, agreed closely, their differences being without statistical significance. In general the Quick prothrombin time values were considerably shorter than those determined by the Howell method. Certain of the June 20 bleedings, however, which were taken after the first hot spell of the season, had Howell prothrombin time values as much as 17 sec. shorter than the Quick prothrombin time values. Since the major difference in the two methods is that thromboplastin is added to the Quick but not in the Howell test, this suggested that these particular June 20 plasmas may have contained such exceptionally large amounts of thromboplastic materials that further addition was not required. Alternatively, there might have been a decrease in a thromboplastin inhibitor. The apparent effect of sudden changes in weather conditions on coagulative behavior seemed of some interest.

Comparison of Complement Titer, Prothrombin Time, and Ascorbic Acid Content

Both the complement titers and the prothrombin time values for the two groups of guinea pigs ranged within what has been considered to be "normal limits". When the sera were divided into three groups on the basis of complement titers, the mean Quick and Howell prothrombin time values were found to be closely comparable for all three categories (Table IV). The higher complement titers of the 14 specimens in the first division were not associated with shorter prothrombin time values nor were the slightly lower complement

TABLE III

A COMPARISON OF THE PROTHROMBIN TIME VALUES DETERMINED BY THE METHODS OF QUICK AND HOWELL FOR PLASMA OF GUINEA PIGS ON THE TWO DIFFERENT DIETS

Date of bleeding, 1949	Number bled	Supplement	Prothrombin time					
			Quick			Howell		
			Range, sec.	Mean, sec.	Standard deviation	Range, sec.	Mean, sec.	Standard deviation
May 2	2	Grass	25 – 27	26.0		41 – 45	43.0	
May 5	3	Grass	24 – 28	25.3		24 – 34	28.6	
June 1	6	Grass	24 – 31	27.9		33 – 61	44.5	
June 2	8	Grass	22 – 31	26.6		38 – 61	47.2	
June 14*	7	Grass	28 – 44	29.7		34 – 48	41.2	
June 15*	4	Grass	24 – 31	29.4		40 – 60	54.5	
June 20*	4	Grass	23 – 39	33.3		20 – 33	26.7	
July 7*	4	Grass	25 – 37	30.1		40 – 67	47.5	
July 19	5	Grass	28 – 42	32.6		43 – 57	47.6	
July 25	7	Grass	26 – 37	31.0		35 – 50	42.1	
May 2	16	Roots	19 – 36	27.7		29 – 59	41.8	
May 5	9	Roots	29 – 41	36.2		30 – 55	43.4	
June 1	5	Roots	26 – 31	29.2		32 – 48	40.6	
June 2	8	Roots	23 – 29	27.5		37 – 45	40.2	
June 15*	4	Roots	15 – 65	34.7		32 – 69	55.0	
June 20*	8	Roots	29 – 50	35.0		21 – 49	39.8	
June 22*	8	Roots	27 – 46	33.3		33 – 46	43.6	
July 7*	4	Roots	26 – 37	31.0		37 – 61	48.7	
July 19	9	Roots	25 – 43	31.1		33 – 53	41 4	
July 25	6	Roots	31 – 39	34.6		42 – 57	49.5	
Mean values	50	Grass	22 – 44	30.0	5.29	20 – 61	42.9	9.89
" "	77	Roots	15 – 65	31.7	7.00	21 – 69	43.3	8.47

* *A period of unusually warm weather for this region.*

TABLE IV

COMPARISON OF COMPLEMENT TITERS AND PROTHROMBIN TIME VALUES

Complement titer,* units/ml.	Number of sera	Prothrombin Time			
		Quick		Howell	
		Range, sec.	Mean, sec.	Range, sec.	Mean, sec.
>2500	14	19 to 39	29.6	20 to 55	36.8
1500 – 2500	87	15 to 65	30.8	21 to 69	44.7
<1500	15	23 to 50	31.6	32 to 49	40.6

* *Minimum titer = 1250 units/ml.*
Maximum titer = 3570 units/ml.

. *The complement titers given are those obtained approximately two hours after bleeding, that is concurrently with or shortly after the coagulation tests were performed.*

titers of the 15 specimens in the third category accompanied by higher prothrombin time values. The factors responsible for these minor variations within the "normal" range were apparently different for the two activities.

In Table V the bleedings have been arranged in six groups according to their plasma – ascorbic-acid levels and independently of diet, although obviously the majority of plasmas in the lower categories were from guinea pigs in the second

TABLE V

A COMPARISON OF PLASMA ASCORBIC ACID CONTENT WITH COMPLEMENT TITERS AND WITH PROTHROMBIN TIME VALUES

Ascorbic acid values, mgm. %	Number of animals	Complement titer				Prothrombin time			
		First day		Second day		Quick		Howell	
		Range, units/ml.	Mean, units/ml.	Range, units/ml.	Mean, units/ml.	Range, sec.	Mean, sec.	Range, sec.	Mean, sec.
0 to 0.19	37	1430 to 3250	1820	1110 to 2330	1520	15 – 65	31.9	21 – 69	43.4
0.20 to 0.59	8	1670 to 3300	2000	1250 to 2000	1420	25 – 42	32.7	22 – 57	43.7
0.60 to 0.99	10	1590 to 2630	2000	1370 to 2000	1560	26 – 37	31.1	33 – 57	43.4
1.00 to 1.39	11	1350 to 3130	2080	1140 to 2220	1470	23 – 37	29.8	39 – 61	49.7
1.40 to 1.79	5	1430 to 2780	1770	1430 to 2000	1640	26 – 44	30.1	34 – 56	42.0
1.80 to 2.85	7	1430 to 2500	2040	1250 to 1670	1520	24 – 38	29.0	20 – 61	39.8

* *Titer expressed in 50% hemolytic units.*

dietary group and all of those in the higher categories from the first dietary group. The range and mean complement titers and prothrombin time values have been assembled and appear closely comparable for each category.

In brief, therefore, although the ascorbic acid content of the respective plasmas had not only differed widely in the two dietary groups, but had also varied significantly within the first group, this was not reflected in any consistent change either in complement titer or in prothrombin time.

Acknowledgments

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THE FRACTIONATION OF PHOSPHORUS CONTAINING CONSTITUENTS IN THE ALLANTOIC MEMBRANE OF THE EMBRYONATED EGG[1]

By A. F. Graham

Abstract

A procedure is described which permitted the phosphorus containing constituents in allantoic membranes of embryonated eggs to be separated into fractions as follows: alcohol soluble phosphorus from which the phospholipids were separated, phosphorus soluble in 5% trichloroacetic acid in which inorganic phosphorus was determined, and nucleic acid phosphorus which was further separated into pentose and desoxypentose nucleic acids. This procedure was applied to membranes between 9 and 13 days of age. It was found that the total phosphorus amounted to 9 to 10 mgm. per gm. of dried tissue. Alcohol soluble phosphorus accounted for approximately 28%, acid soluble phosphorus for 37%, and nucleic acid phosphorus for 35% of the total phosphorus. About half the acid soluble phosphorus was inorganic and about 85% of the alcohol soluble phosphorus was associated with phospholipids. These proportions remained essentially constant over the period studied. The ratio of pentose to desoxypentose nucleic acid phosphorus also remained fairly constant over the interval at about 2.2. There was no significant difference in the amounts of P^{32} taken up over a period of 72 hr. by normal allantoic membranes and those infected with influenza virus, when inorganic radioactive phosphorus was placed in the allantoic sacs of 11-day embryonated eggs.

Introduction

Previous work has demonstrated (3) that influenza virus A growing in the allantoic membrane of the embryonated egg in the presence of radioactive inorganic phosphorus incorporated the isotope into its structure. Both the nucleic acid and phospholipid components of the virus were found to contain the isotope (2).

In view of these findings the question arose as to whether nucleic acid and phospholipid already present in the cell were incorporated into the virus or whether these components of the virus were synthesized after infection of the cell. As an aid in answering this question, it was considered that some information should be obtained on the rate of uptake of P^{32} by the constituents of normal cells.

Prior to this study it was necessary to determine the distribution of phosphorus among the various components of allantoic membrane. The present paper describes the techniques employed to separate the phosphorus of allantoic membrane into fractions soluble in alcohol, 5% trichloroacetic acid, and a residue containing nucleic acid and phosphoprotein. Further, the phospholipid present in the alcohol soluble fraction, inorganic phosphorus in the trichloroacetic acid soluble fraction, and the proportion of pentose to desoxypentose nucleic acid were estimated. This procedure, which, in many respects,

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From Connaught Medical Research Laboratories, University of Toronto, Canada. Aided by a grant from the W. B. Boyd Memorial Fund.

is similar to that described by McCarter and Steljes (4) for rat tissue, was applied to membranes collected from fertile eggs 9 to 13 days of age and the results are described.

Some preliminary results on the uptake of P^{32} by allantoic membranes before and after infection with influenza virus are also described.

Methods

Estimation of Total Phosphorus

The method for determining total phosphorus has been described in a previous paper (3).

Estimation of Radioactive Phosphorus

A liquid Geiger-Müller counter, capacity 10 ml., as described by Veall (7) was used in conjunction with a conventional scale of 64 unit. The counter was calibrated against a standard Ra D + E source from the National Bureau of Standards, Washington, as previously described (2). In this paper the activities of radioactive sources are recorded as counts per minute (c.p.m.) 1000 c.p.m. being equivalent to 86 μrd.

Preparation of Allantoic Membranes for Analysis

Embryonated eggs from a pure bred White Leghorn flock, previously candled to ensure that they were alive, were opened at the air sac end, an incision was made in the allantoic membrane and the contents of the egg were poured out. Generally the allantoic membrane was left adhering to the inside of the egg; it was rapidly removed, rinsed twice in ice-cold 0.85% sodium chloride (w/v) immediately frozen and stored at −20° C. in a screw capped vial. Before use the material was immersed in liquid nitrogen in a stainless steel mortar, pulverized, dried *in vacuo* from the frozen state and stored over P_2O_5 in a vacuum desiccator. No attempt was made to choose eggs of a particular weight.

Experimental

Extraction of Trichloroacetic Acid Soluble Phosphorus and Alcohol Soluble Phosphorus from Membrane Tissue

To determine whether extraction of dried membrane with 5% trichloroacetic acid (TCA) would influence later extraction of alcohol soluble phosphorus, an experiment was performed in which one quantity of tissue was extracted with boiling alcohol and then with 5% TCA and a second quantity was extracted with 5% TCA followed by extraction with hot alcohol.

Finely powdered tissue, 0.232 gm., was extracted with 5 ml. of boiling absolute alcohol (freshly purified by refluxing over solid potassium hydroxide and then distilling through a fractionating column) for five minutes in a centrifuge tube fitted with a small reflux condenser. After centrifuging the supernatant solution was removed and made to 25 ml. volume. This procedure was repeated twice more on the residue, and phosphorus estimations were carried out on each extract.

The extracted residue was allowed to dry at 37° C. and homogenized in 5 ml. of ice-cold 5% TCA for two minutes in a homogenizer of the type described by Potter and Elvehjem (5). During this procedure the homogenizer was surrounded with crushed ice. The mixture, with 2 ml. of 5% TCA used to wash out the grinder, was centrifuged and the supernatant made to 25 ml. volume. The residue was extracted twice more with 5% TCA in the same manner and phosphorus analyses were carried out on each extract.

In the second part of the experiment the above extraction sequence was reversed. The tissue, 0.232 gm., was extracted three times with 5 ml. of 5% TCA as above. The residue was resuspended in 5 ml. of absolute alcohol to remove residual TCA, centrifuged, and this was repeated. The two alcohol washes were combined and made to 25 ml. This solution is designated "1st alcohol extract". Two further extractions of the tissue with boiling absolute alcohol for five minutes each were carried out and the three extracts were analyzed for phosphorus.

The results of this experiment are shown in Tables I and II. It is seen that the amount of phosphorus extracted from the tissue by boiling alcohol or 5%

TABLE I

AMOUNTS OF PHOSPHORUS PRESENT IN SUCCESSIVE ALCOHOL EXTRACTS OF ALLANTOIC MEMBRANE BEFORE AND AFTER EXTRACTION WITH TRICHLOROACETIC ACID, 0.232 GM. DRY TISSUE USED IN EACH CASE

Fraction	Alcohol extracts before TCA extraction of tissue, μgm. P	Alcohol extracts after TCA extraction of tissue, μgm. P
1st extract	380	373
2nd extract	53	66
3rd extract	14	2

TABLE II

AMOUNTS OF PHOSPHORUS PRESENT IN SUCCESSIVE TRICHLOROACETIC ACID EXTRACTS OF ALLANTOIC MEMBRANE BEFORE AND AFTER EXTRACTION OF THE TISSUE WITH ALCOHOL, 0.232 GM. DRIED TISSUE USED IN EACH CASE

Fraction	5% TCA extracts before alcohol extraction of tissue, μgm. P	5% TCA extracts after alcohol extraction of tissue, μgm. P
1st extract	525	508
2nd extract	40	35
3rd extract	7	5

TCA was the same regardless of which solvent was employed first. Further, three extractions of the tissue with either solvent were sufficient to remove all the phosphorus compounds soluble in that solvent. This observation was

amply confirmed in other experiments. In some experiments a mixture of alcohol and ether (3/1, v/v) was used instead of absolute alcohol alone with similar results.

During extraction with TCA in the glass homogenizer a considerable amount of glass was ground off and became mixed with the tissue. Control experiments showed that this glass did not interfere in the phosphorus estimation.

Separation of Phospholipids from the Alcohol Soluble Fraction of Allantoic Membrane

An aliquot of the alcohol extract of tissue was evaporated to dryness at 30° C. in a stream of nitrogen. The residue was extracted three times for 10 min. each with warm petroleum ether (b.p. 40° – 60° C.) and the extracts, containing the phospholipids, were combined and analyzed for phosphorus.

To determine whether extraction of the tissue with 5% TCA prior to extraction with alcohol influenced the separation of phospholipid, the above procedure was applied to two alcohol extracts of membrane. The first alcohol extract was obtained after the tissue had been extracted with 5% TCA and the second was prepared directly from dried tissue. In both cases phosphorus analyses were carried out on the petroleum ether soluble fraction (phospholipid) and on the petroleum ether insoluble residue, the results being shown in Table III.

TABLE III

SEPARATION OF PETROLEUM ETHER SOLUBLE PHOSPHORUS FROM ALCOHOL SOLUBLE FRACTION OF DRIED ALLANTOIC MEMBRANES

Fraction	Alcohol extract prepared before TCA extraction of membranes, μgm. P	Alcohol extract prepared after TCA extraction of membranes, μgm. P
Petroleum ether soluble	34.2	20.2
Petroleum ether insoluble	8.0	22.0
Total alcohol soluble P added	45.7	44.7

It would appear that separation of phospholipid was less complete in the case where the membrane had been treated with TCA prior to alcohol extraction. It is noteworthy that this alcohol extract contained three to four times the amount of solid material found in the other alcohol extract. It was concluded that the phospholipids were more difficult to separate from the alcohol soluble material when the tissue had been subjected to previous treatment with TCA.

Estimation of Inorganic Phosphate in Trichloroacetic Acid Extracts of Membrane

In estimating inorganic phosphorus in trichloroacetic acid extracts of tissue the usual colorimetric method (3) was applied to the extracts without prior

acid digestion. To determine whether the sulphuric acid and molybdate present in the reagent might liberate inorganic phosphorus from easily hydrolyzable compounds in the extracts, the following experiment was performed.

A small amount of dried tissue was extracted in the cold with 5% TCA. Aliquots of the extract were added to three tubes containing molybdate and sulphuric acid, in the proportions required by the method, and the volumes made up to about 13 ml. with water. The color was developed in the first tube immediately by addition of stannous chloride solution and in the other tubes when they had stood 15 min. and 30 min. respectively at room temperature. The amounts of phosphorus estimated in the three tubes were identical.

A further experiment was carried out to find whether inorganic phosphorus might be liberated by the action of TCA during preparation of the extracts. A small quantity of tissue was extracted as rapidly as possible in the cold with 5% TCA. After centrifuging in the cold an aliquot was taken immediately from the supernatant solution for estimation of inorganic phosphorus. The remaining extract was kept at 37° C. and estimations of inorganic phosphorus were carried out at intervals over a period of 90 min. There was no change in the amount of inorganic phosphorus estimated over this period.

As a further check, the inorganic phosphorus contents of several TCA extracts of tissue were estimated using the modification of the method of Delory (1) described in a previous paper (2). Inorganic phosphorus was also estimated directly in these extracts as described above. On the average the amount of inorganic phosphate found from the direct estimation was about 10% higher than that found with Delory's method.

Estimation of Pentose Nucleic Acid (PNA) and Desoxypentose Nucleic Acid (DNA) in Allantoic Membrane

The method used was that of Schmidt and Thannhauser (6) and as applied in the present work was as follows. To tissue (0.100–0.250 gm.) which had been extracted with alcohol and 5% TCA, as described in preceding sections, was added 4.0 ml. of 1.0 *N* potassium hydroxide. The mixture was incubated 18–20 hr. at 37° C., during which time the tissue went into solution, and was then centrifuged to remove ground glass resulting from the preliminary TCA extraction in the homogenizer. Aliquots of supernatant were taken for estimation of total nucleic acid phosphorus. To 3 ml. of supernatant was added 3 ml. of 5% TCA and 0.6 ml. of 6 *N* hydrochloric acid. The precipitated DNA and protein was centrifuged off and washed once with 2.5 ml. of 5% TCA. This wash was added to the first supernatant, the total volume was made to 10 ml. and phosphorus analyses were carried out to determine PNAP. About 1 ml. of 0.1 *N* potassium hydroxide was added to the DNA precipitate which was dissolved by warming, the volume was made up to 10 ml., and the DNAP content was estimated by phosphorus analysis.

To determine the efficiency of the method in separating DNA and PNA, two samples of lipid free, TCA extracted tissue of equal weight were taken.

To one of these were added known amounts of calf thymus nucleic acid and yeast nucleic acid. Both samples were submitted to the separation procedure described above. The results are shown in Table IV.

TABLE IV

SEPARATION OF YEAST NUCLEIC ACID AND THYMUS NUCLEIC ACID, ADDED TO LIPID FREE, TRICHLOROACETIC ACID EXTRACTED ALLANTOIC MEMBRANE TISSUE, BY THE METHOD OF SCHMIDT AND THANNHAUSER

	Total phosphorus, μgm.	PNA phosphorus, μgm.	DNA phosphorus, μgm.
Membrane (0.118 gm.) with added PNA and DNA	468	297	168
Membrane (0.118 gm.) alone	324	230	92
Recovered	144 (94%)	67 (93%)	76 (94%)
Added	153	72	81

It is seen that the recovery of added PNAP and DNAP was 93 to 94% of that added.

On several occasions DNAP and PNAP, after separation by the Schmidt and Thannhauser method, were estimated by the diphenylamine and orcinol color reactions as previously described (2). The diphenylamine reaction was applied after the DNA had been extracted from the protein with 5% TCA for 45 min. at 95° C. This extraction removed all but 3% of the phosphorus of the DNA fraction.

The results obtained with these color tests varied a good deal, the orcinol reaction indicating 15 to 35% more PNAP than direct phosphorus estimation and the DNAP being 12 to 25% higher by the diphenylamine reaction than by phosphorus analysis.

Distribution of Phosphorus Constituents in Allantoic Membranes

As a result of the above experiments the following procedure was adopted to fractionate the phosphorus containing constituents of allantoic membranes.

The dry weighed material, 100 to 350 mgm., was extracted with boiling absolute ethanol, or ethanol–ether (3/1, v/v), phosphorus analyses being carried out on the extract. An aliquot of the alcohol extract was evaporated to dryness and thoroughly extracted with petroleum ether, to remove phospholipids, and this solution was analyzed for phosphorus. The alcohol insoluble material was homogenized with 5% TCA, total and inorganic phosphorus being estimated in the extract. The Schmidt and Thannhauser technique was applied to the residue to separate the nucleic acids. PNAP as estimated here includes the phosphorus from any phosphoprotein which may have been present in the membrane, no attempt being made to estimate the phosphoprotein separately.

This analytical procedure was applied to membranes collected at 24 hourly intervals from embryonated eggs 9 to 13 days of age. Seven or eight membranes were collected at each time, pooled, and analyzed together. The results are shown in Figs. 1, 2, and 3. In Fig. 1 the phosphorus contents of the various

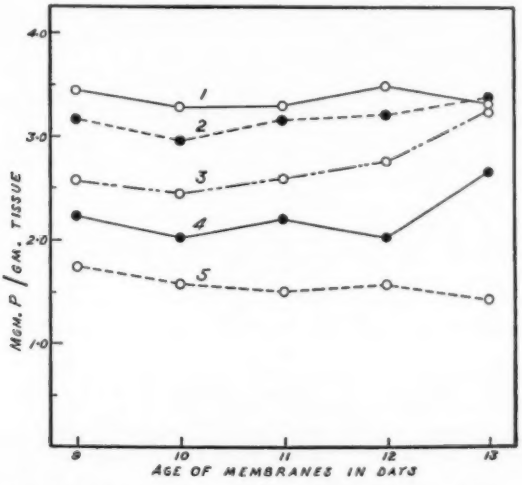

FIG. 1. *Phosphorus contents in mgm. per gm. of dry tissue of the various fractions of allantoic membranes between 9 and 13 days of age. Curve 1, 5% trichloroacetic acid soluble phosphorus. Curve 2, total nucleic acid soluble phosphorus. Curve 3, alcohol soluble phosphorus. Curve 4, petroleum ether soluble phosphorus (phospholipid). Curve 5, inorganic phosphorus, present in 5% trichloroacetic acid soluble fraction.*

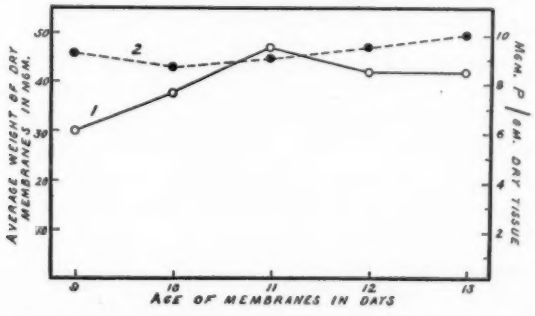

FIG. 2. *Relationship of average dry weight of allantoic membranes, Curve 1, and total phosphorus content, Curve 2, to age of membranes.*

fractions calculated in terms of mgm. P per gm. dry tissue are plotted against the age of the membrane in days. In Fig. 2 are plotted the average weights of the membranes in mgm. and the total phosphorus content of the membranes as mgm. per gm. dry tissue. The figures for the total phosphorus contents of the membranes were obtained by addition of the results from the individual

fractions shown in Fig. 1. Fig. 3 shows the ratio of PNAP (including phosphoprotein phosphorus) to DNAP, obtained in three separate experiments, plotted against age of membrane.

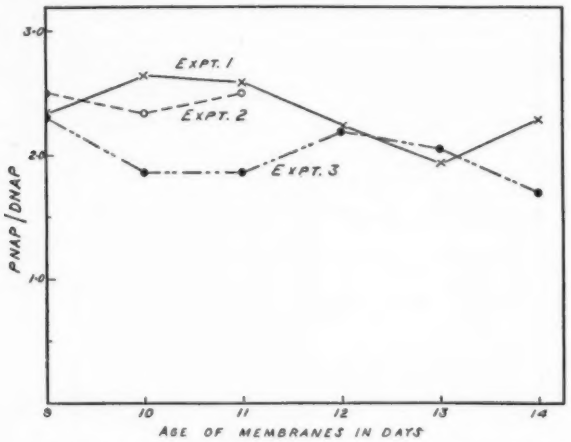

FIG. 3. *The ratio of pentose nucleic acid phosphorus, PNAP, to desoxypentose nucleic acid phosphorus, DNAP, in allantoic membranes between 9 and 14 days of age.*

The results shown in Figs. 1 and 2 for a single experiment were similar to those obtained in a second complete experiment carried out on membranes 9 to 14 days of age.

Uptake of Radioactive Phosphorus by Normal Allantoic Membranes and Membranes Infected with Influenza Virus A

The general procedure for determining the uptake of P^{32} by allantoic membranes was as follows. P^{32}, as inorganic phosphate in 0.85% sodium chloride solution, was injected from a calibrated syringe into the allantoic fluid of 25 to 30 11-day embryonated eggs, 0.2 ml. containing 3000 to 4000 c.p.m. into each egg. The eggs were then incubated at 36° C. and at intervals the membranes from a group of five or six eggs were collected. After being washed thoroughly in two changes of ice-cold saline, the membranes were digested in a mixture of 5 ml. of concentrated sulphuric acid and 2 ml. of 72% perchloric acid and the volume was made up to 50 ml. Radioactivity estimations were carried out on these solutions.

In one experiment with 11-day-old embryonated eggs the uptake of P^{32} by normal membranes was determined over a period of 115 hr. and the results are shown in Fig. 4 as Curve 3. A second experiment was carried out in which two groups of 25 11-day eggs were chosen at random from a large number. Influenza virus A (PR8 strain) was injected in 0.2 ml. amount (10000 ID_{50}) by the allantoic route into each egg of the first group. P^{32} was then injected

into all the eggs which were incubated at 36° C. The P^{32} contents of the membranes of each group were determined at intervals as described above and the results are plotted in Fig. 4, as Curves 1 and 2.

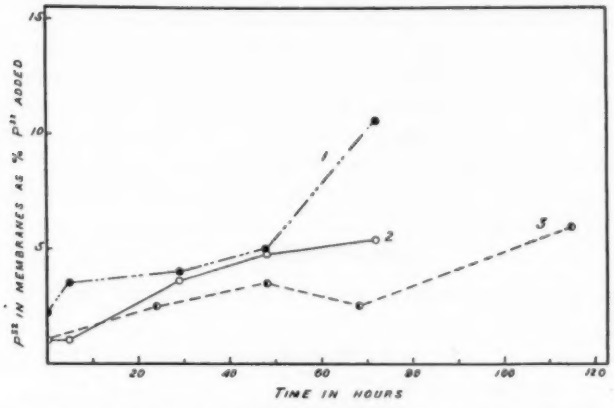

FIG. 4. *Uptake of P^{32} by allantoic membranes from inorganic radioactive phosphorus placed in the allantoic sacs of 11-day embryonated eggs. Curve 1 represents uptake of P^{32} for eggs infected with influenza virus and Curve 2 for normal embryonated eggs, the experiments being carried out simultaneously. Curve 3 represents uptake of P^{32} by normal membranes in a separate experiment.*

Discussion

The procedure described permitted the phosphorus constituents of allantoic membrane to be separated into several fractions, acid soluble phosphorus in which the inorganic phosphorus was determined, alcohol soluble phosphorus from which the phospholipids were separated, and nucleic acid phophorus which was further divided into PNAP and DNAP.

When this procedure was applied to membranes between 9 and 13 days of age the amounts of acid soluble, inorganic, alcohol soluble, phospholipid, nucleic acid, and total phosphorus per gram of tissue remained essentially constant over this interval. Acid soluble P accounted for about 37%, alcohol soluble P for about 28%, and nucleic acid P for about 35% of the total phosphorus of the membrane. In a second such experiment the figures were 46%, 21%, and 33% respectively. The total phosphorus in the membranes amounted to 9 to 10 mgm. per gm. of dried tissue. About half the TCA soluble P was inorganic and about 84% of the alcohol soluble P was contained in phospholipids. Since the membranes on the average increased about 80% in weight between 9 and 11 days the actual amounts of the various phosphorus constituents increased by roughly the same proportion.

Although there was considerable variation in the PNAP/DNAP ratio this ratio may be greatly influenced by small errors in analysis and it is considered that it remains essentially constant at about 2.2 over the 9 to 14 day period.

It is doubtful whether there was any significant difference between the amounts of P^{32} taken up by normal and virus infected 11–day membranes. However, further work may show that there is a significant difference in the rates with which the various components of the membrane are labelled in the two cases. It would be of interest to determine whether the rate of uptake is faster by nine-day membranes than by those 11 days of age since the membrane weight and phosphorus content increase roughly twofold between 9 and 11 days. It seems possible that the P^{32} in the allantoic fluid is not readily utilized by the membrane cells. If this is so, it would be worthwhile to determine the main source of the phosphorus utilized by these cells. A method might then be devised for increasing the specific radioactivity of influenza virus grown in the membrane.

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STUDIES ON THE RELATIONSHIP BETWEEN VIRUS AND HOST CELL: THE PREPARATION OF T2r^+ BACTERIOPHAGE LABELLED WITH RADIOACTIVE PHOSPHORUS[1]

By S. M. Lesley, R. C. French, and A. F. Graham

Abstract

T2r^+ bacteriophage grown in its host, *Escherichia coli* B, in broth medium in the presence of radioactive inorganic phosphorus was labelled with the isotope. Purified suspensions of this virus had specific activities up to 50,000 c.p.m. per μgm. P. There was little or no exchange of P^{32} between virus and inorganic phosphate. Chemical analysis showed that at least 98% of the virus phosphorus was contained in nucleic acid; of the nucleic acid phosphorus 95.5% was associated with desoxypentose nucleic acid and 4.5% with pentose nucleic acid. More than 99% of the radioactivity of the labelled bacteriophage was contained in the nucleic acid fraction. Preparations of bacteriophage were obtained with sufficiently high specific activity to enable metabolism experiments to be carried out on the growth of the labelled virus in the host cell.

Introduction

In a previous paper (6) a method was described which enabled influenza virus growing in the embryonated egg to be labelled with radioactive phosphorus. As was pointed out the main purpose of that work was to obtain highly radioactive specimens of influenza virus to enable the metabolism of the isotope to be studied when cells were infected with the labelled virus. While little difficulty was experienced in labelling influenza virus with relatively small amounts of P^{32}, the specific activity was not sufficiently high to enable the metabolism experiments to be carried out.

It was considered, however, that such a study might be more feasible with a different virus – host cell system, namely T2 bacteriophage active on *Escherichia coli*, strain B. It has already been demonstrated by Cohen (1) that the T2 and T4 bacteriophage of *E. coli*, and by Kozloff and Putnam (10) that the T6 bacteriophage were labelled with P^{32} when inorganic radioactive phosphorus was added to the medium supporting virus growth in the infected cells.

The present paper describes the methods used to obtain purified highly radioactive preparations of T2r^+ bacteriophage labelled with P^{32}. Experiments are described which support the conclusion that the label is contained almost entirely in the nucleic acid fraction of the virus.

Utilizing these methods preparations of bacteriophage were obtained sufficiently high in specific radioactivity to enable metabolism experiments to be carried out with the virus on its host, *E. coli*, strain B.

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Methods and Materials

Determination of Total Phosphorus

The method of estimating total phosphorus has been previously described in detail (6).

Determination of Radioactive Phosphorus

The radioactivity of P^{32} solutions was estimated with a liquid Geiger-Müller counter, capacity 10 ml., of the type described by Veall (13). The counter was standardized against a RaD+E source from the National Bureau of Standards, Washington, as previously described (5). The results of radioactivity measurements, given in this paper in terms of counts per minute (c.p.m.), may be converted to disintegrations per second using the factor 1000 c.p.m. are equivalent to 86 μrd.

Specific activity is defined as c.p.m. per μgm. P unless otherwise indicated.

Preparation of Media

The liquid medium used throughout this work was a nutrient solution prepared by dissolving 20 gm. Difco Bacto–Tryptose, 5 gm. sodium chloride, and 1 gm. glucose per liter in distilled water. After adjusting the pH to about 7.2, the solution was autoclaved. Solid medium was prepared by adding agar to the broth medium before autoclaving.

Type of Bacteriophage and Bacteria

The bacteriophage used throughout was the T2r^+ strain, active on *E. coli* B, received from Dr. Fred Heagy, University of Western Ontario. Using the method of Delbrück and Luria (3) this bacteriophage was found to have a latent period of 23 min. and a burst size of 115 in Bacto–Tryptose broth.

Determination of Bacterial Concentration in Liquid Cultures

The concentration of cells in broth culture was estimated on a Coleman Spectrophotometer at 630 mμ, the per cent transmittance being read against broth as a blank. The instrument was standardized against cell suspensions in which the number of cells was determined by colony count and also by direct count in a Petroff–Hausser counting chamber.

Determination of Virus Concentration

The number of infective particles in a bacteriophage suspension was determined by the method of Hershey *et al.* (7). Since slight modifications were made to the original method, the procedure is described below.

Serial 10- or 100-fold dilutions of the virus solution were made in 0.85% (w/v) sodium chloride (saline) until the number of infective centers in the final dilution was between 60 and 1200. To 1 ml. of this dilution was added 3 ml. of broth suspension of *E. coli* containing about 2×10^8 cells per ml., freshly prepared from a 15–20 hr. agar slope. One ml. aliquots of this mixture were then added to each of three tubes containing 1 ml. of melted nutrient agar (0.7%). The contents of each tube were mixed and poured over the surface

of a nutrient agar (1%) plate. When the agar had solidified the plates were incubated for 18 hr. at 37° C. and the plaques were counted. From the results the number of plaque forming particles per ml. of the original suspension was calculated and in this paper is expressed as phage per ml. The plating efficiency determined by the method of Ellis and Delbrück (4) was about 0.65.

When titrating saline suspensions of bacteriophage, prepared as described in a later section, a 100-fold dilution of the suspension was made in broth and allowed to stand at least one hour before further dilutions were made in saline. It was found that higher titers were obtained by this procedure than when the initial dilution was made in saline.

Experimental

Growth and Purification of Bacteriophage

Cultures of *E. coli* B were prepared by inoculation of 150 ml. of broth from an 18 hr. slope and grown to 2×10^8 cells per ml. with rapid aeration at 37° C. The generation time of *E. coli* under these conditions was about 21 min. After centrifuging, the cells were resuspended in 150 ml. of fresh broth at 37° C. and immediately inoculated with T2r^+ bacteriophage from a stock broth culture usually in the ratio of three to five virus particles per cell. In some cases, for example in preparing phage labelled with P^{32} as described later, the centrifuging and resuspension of the cells were omitted and the virus inoculum was added to the culture as soon as it has reached the required concentration of cells.

The culture was rapidly aerated at 37° C. until lysis occurred when the titer of phage reached 4×10^{10} to 10^{11} phage per ml. Bacterial debris was then removed by centrifuging at 4300 g. for 30 min. in a Sorvall angle centrifuge at 5° C. About 25% of the virus was lost in this step, but further purification depended upon efficient removal of the debris. The virus was then sedimented by centrifuging the supernatant solution at 22,000 g. for one hour in the angle centrifuge. The supernatant was aspirated off until the level of liquid was just above the virus pellet which adhered to the side of the tube. When the tube was left for some time a bluish material was observed to separate slowly from the pellet and fall to the bottom of the tube. By this means a separation was obtained between virus and the nonviral material which remained adhering to the tube as an opaque tightly packed pellet. When the separation was complete, after 20–40 min., as much as possible of the remaining liquid and the pellet were carefully drawn off through a fine tipped pipette leaving the virus in the bottom of the tube as a small opalescent pool. A few ml. of 0.85% saline were then added and the virus was dispersed by giving the tube a quick swirl. The volume was made up with saline to about one-quarter the original volume, the virus was sedimented at 22,000 g. and separated from the pellet as before. The nonviral pellet was very small at this stage and was absent when the procedure was repeated a third time. The virus was finally resuspended in 10 ml. of saline, the recovery being 25 to 40% of that

present in the original lysate. With this procedure the customary intermediate low speed centrifugations to remove aggregated material after resuspension of the virus were unneccessary.

Properties of the Purified Virus

Preparations of purified virus in 0.85% saline containing 10^{11} to 4×10^{12} phage per ml. gave single boundaries in the ultracentrifuge (Fig. 1) with an average sedimentation constant of 1040 S in agreement with the results of Sharp *et al.* (11).

Chromium shadowed preparations in the electron microscope showed the usual tadpole-shaped particles (8), the dimensions of the head being approximately 90 by 125 mμ, the length of the tail about 110 mμ.*

Phosphorus analyses carried out on six preparations of purified phage gave 2.76, 2.67, 4.15, 2.94, 2.15, 3.46×10^{-11} μgm. P per phage or an average of 3.02×10^{-11} μgm. P per phage.

Saline suspensions of purified bacteriophage, kept at 5° C., did not decrease in titer over a period of eight weeks.

Preparation of Bacteriophage Labelled with Radioactive Phosphorus

In order to obtain virus of high specific activity the phosphorus content of the broth medium was decreased as follows. Twenty gm. of Bacto–Tryptose was dissolved in about 200 ml. of distilled water, the solution was adjusted to pH 8.4 and 2 ml. saturated calcium chloride solution was added. The precipitate of calcium phosphate was removed by centrifugation, sodium chloride and glucose were added in the required proportions to the supernatant solution, the volume was made to 1000 ml., and the solution autoclaved. By this means the total phosphorus content of the final medium was reduced from 140 to about 30 μgm. P per ml.

To 150 ml. of this medium was added up to 1.5 mc. of P^{32} as inorganic phosphate, carrier free. The medium was inoculated with *E. coli* from an 18 hr. slope and grown to 2×10^8 cells per ml. at 37° C. with aeration. Bacteriophage from a stock culture was then added to give a final concentration of 6 to 8×10^8 phage per ml., and the suspension was aerated until lysis occurred.

The bacteriophage, which reached a final titer of 4×10^{10} to 10^{11} phage per ml. in the lysate, was purified in the manner previously described. The virus was sedimented repeatedly, usually four to six times, until the specific activity of the supernatant solution, c.p.m. per phage, reached that of the resuspended virus. After each sedimentation the phage was resuspended in about 40 ml. of saline the final suspension being made in 8 to 12 ml. of saline. By this means suspensions of purified virus were obtained having titers of 1 to 2×10^{11} phage per ml., and specific activities varying from 500 to 50,000 c.p.m. per μgm. P depending on the amount of P^{32} added to the medium.

* *We are indebted to Dr. G. D. Scott of the Physics Department for the electron micrographs.*

PLATE I

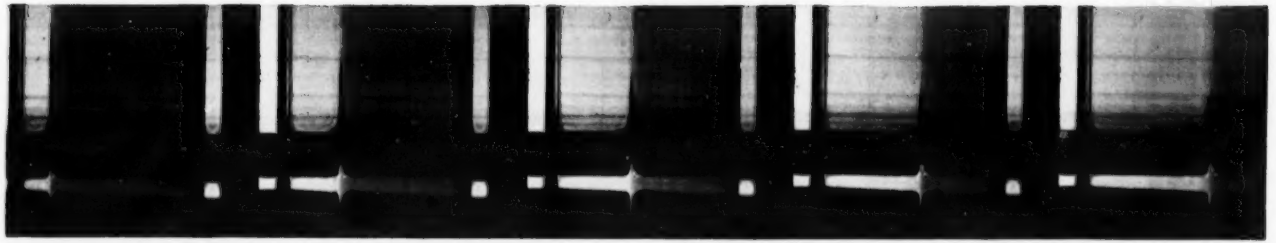

FIG. 1. *Sedimentation diagrams of purified T2r⁺ bacteriophage in 0.85% sodium chloride at pH 6.05. Concentration was 1.76 mgm. phage per ml. Direction of sedimentation is from left to right. Photographs were taken at two-minute intervals with a mean centrifugal field of 11,100 g. using a Spinco Ultracentrifuge equipped with a Philpot-Svensson type of optical system.*

Since these radioactive preparations were required for other experiments phosphorus analyses were carried out on only a small number. However, there appeared to be no difference in the μgm. P per phage between normal and radioactive preparations. Further, repeated phage titrations carried out at intervals over a period of several weeks with several highly radioactive phage preparations showed no inactivation of virus by the radiation.

Control Experiments to Determine Whether Bacteriophage Takes up P^{32} by Exchange with Inorganic Phosphate

To determine whether P^{32} was taken up by bacteriophage by exchange or adsorption on its surface the following experiment was carried out.

To two tubes, each containing 150 ml. of broth culture of *E. coli*, in the exponential phase of growth, at a concentration of 5×10^7 cells per ml., was added sufficient T2r^+ bacteriophage to give a ratio of approximately 1 virus particle per 40 cells. P^{32} was added immediately to tube A to give a final concentration of 75,000 c.p.m. per ml. Both cultures were then aerated at 37° C. until lysis occurred. An identical amount of P^{32} was then added to tube B as had been added to tube A and both cultures were allowed to stand overnight at 5° C. The concentration of virus in both cultures at lysis was about 7.5×10^{10} phage per ml. The phage was then purified as previously described and total phosphorus and radioactivity estimations were carried out on the final virus suspensions. Purified phage from tube A had a specific activity of 481 c.p.m. per μgm. P, while that from tube B had 1.6 c.p.m. per μgm. P. It was thus apparent that by far the greater proportion of P^{32} contained in radioactive phage was incorporated during growth of the virus in the host cells.

In a further experiment 1.0 ml. of P^{32} (carrier free) solution in saline was added to 1.0 ml. of purified phage to give a final P^{32} concentration of 326,000 c.p.m. per ml. and a specific activity of 28,200 c.p.m. per μgm. P. After standing for 18 hr. at 5° C. the virus was alternately sedimented at 22,000 g. and resuspended in 40 ml. of saline. After the fifth sedimentation the specific activity was found to be 209 c.p.m. per μgm. P, and after the sixth 82 c.p.m. per μgm. P. The final specific activity of the virus was thus 0.3% of the specific activity contained in the initial suspension, indicating little or no exchange or adsorption of radioactive inorganic phosphorus by the virus.

Further Tests to Determine Whether P^{32} was Incorporated into the Structure of Radioactive Bacteriophage

It was thought that if a significant proportion of the P^{32} contained in radioactive bacteriophage were adsorbed on the surface it might be released when the phage was adsorbed on the host cells. To investigate this point purified radioactive phage in broth was added to a broth suspension of *E. coli* to give a ratio of one virus particle to two cells with the final concentration of cells being about 10^{10} per ml. The mixture was kept at 37° C. for seven minutes then chilled to 5° C. and centrifuged to remove the cells and adsorbed bacteriophage. It was found that the supernatant solution thereby obtained contained

14% of the added phage and 16% of the added P^{32}. For all radioactive phage preparations so far tested by this method similar results have been obtained. The isotope would appear to be firmly fixed to the virus particle.

Since trichloroacetic acid is widely used as a solvent to extract inorganic and low molecular weight phosphorus compounds from cells it was of interest to determine whether purified radioactive phage contained phosphorus compounds soluble in trichloroacetic acid. Accordingly, a quantity of purified radioactive phage was added to 20 ml. of broth and allowed to stand an hour or so to ensure that the virus was dispersed. The solution was chilled in an ice bath and 0.2 ml. of dialyzed 1% egg albumin solution was added to act as a protein carrier, followed by 2.9 ml. of 40% trichloroacetic acid. After standing for 15 min. in the cold the precipitate was removed by centrifuging and the P^{32} content of the supernatant determined.

This procedure was carried out on each of nine preparations of purified radioactive phage. The amount of radioactivity remaining in the trichloroacetic acid supernatant was generally less than 0.5% of that added originally with the bacteriophage. In the case of one preparation 0.9% of the added radioactivity was soluble in the trichloroacetic acid. In this particular case a suspension of the purified phage in broth was dialyzed for several days against several changes of 0.1 *M* phosphate buffer pH 7.0. The dialyzate was found to contain 0.9% of the radioactivity present in the bacteriophage.

It is evident from these results that the preparations of purified bacteriophage contained little P^{32} soluble in trichloroacetic acid suggesting that the isotope was incorporated in the structure of the virus.

Chemical Analysis of Radioactive Bacteriophage

In order to obtain additional evidence that the bacteriophage was labelled, a chemical analysis was carried out on a purified radioactive specimen as follows: A preparation of purified radioactive bacteriophage suspended in saline was diluted with about eight times its quantity of purified but not radioactive phage. This gave a suspension containing sufficient virus (about 4.5 mgm.) for analysis and containing an amount of activity convenient for measurement. The preparation contained 2.7×10^{-11} μgm. P per phage and a specific activity of 657 c.p.m. per μgm. P. To the suspension was added 0.25 ml. of 1% egg crystalline albumin to act as a protein carrier. An analytical control, which was treated throughout in the same manner as the virus, contained 0.25 ml. of the albumin solution in saline.

Sufficient 40% trichloroacetic acid was added to give a final concentration of 5% and the precipitate was removed by centrifugation. The residue was washed twice with cold absolute alcohol and then extracted twice with boiling alcohol–ether (3/1, v/v) for 15 min. The residue was then extracted three times at 95° C. with 5% trichloroacetic acid for 15 min. to remove the nucleic

acid. P^{31} and P^{32} estimations were carried out on the 5% trichloroacetic acid, alcohol–ether, and nucleic acid fractions and the nucleic acid free residue; the results are shown in Table I.

TABLE I

CHEMICAL ANALYSIS OF RADIOACTIVE BACTERIOPHAGE

Fractions	Total P^{31}, μgm.	Total P^{32}, c.p.m.	Specific activity, c.p.m. per μgm. P
Whole virus	189	124000	657
5% Trichloroacetic acid soluble	4.2	1175	280
Alcohol-ether soluble	0	50	—
Nucleic acid	210	137000	653
Nucleic acid free residue	0	346	—

It is observed that the phosphorus of the virus is contained almost entirely in the nucleic acid fraction in accordance with the observations of Taylor (12). Further, practically all the radioactivity is associated with the nucleic acid.

Since Taylor (12) has reported the presence of 6.6% pentose nucleic acid and 40.3% desoxypentose nucleic acid in this virus, the orcinol reaction for pentose and the diphenylamine reaction for desoxypentose were applied to the nucleic acid fraction of the virus in the manner described in a previous paper (5). The results of these tests showed the presence of 9.8 μgm. of pentose nucleic acid phosphorus and 204 μgm. of desoxypentose nucleic acid phosphorus, compared to 210 μgm. P in the fraction (Table I) estimated by phosphorus analysis.

Discussion

From sedimentation and electron microscope studies it is concluded that the preparations of purified T2r^+ bacteriophage obtained in this work consisted almost entirely of the virus particles. This conclusion is supported by the finding that one plaque forming unit in the preparations contained about 3.0×10^{-11} μgm. P, a result not inconsistent with the figure of 4.7×10^{-11} μgm. P per phage calculated from the results of Hook *et al.* (8) for this virus and 3.9×10^{-11} μgm. P per phage found by Kozloff and Putnam (9) for the closely related T6r^+ bacteriophage of *E. coli*.

At least 98% of the phosphorus of the virus was contained in the nucleic acid fraction, the remaining 2% being soluble in 5% trichloroacetic acid. About 95.5% of the nucleic acid phosphorus was found in desoxypentose nucleic acid and about 4.5% in pentose nucleic acid. While these results confirm the observation of Taylor (12) that both nucleic acids are present in purified preparations of the virus yet recent work by Cohen (2) indicates that the PNAP found may be present as an impurity. The amount of pentose nucleic acid phosphorus determined by Taylor for this virus by a different method, namely 12.7% of the total nucleic acid phosphorus, was somewhat

higher than obtained in the present work. Kozloff and Putnam (9) have found 2.4 to 3.4% of the total phosphorus in synthetic medium T6r^+ bacteriophage to be present in the nucleic acid.

Sufficient P^{32} was introduced into the virus during its growth to enable metabolism experiments to be carried out with the labelled phage growing on the host cells. That the isotope was, in fact, incorporated into the structure of the virus was indicated by control experiments which showed little or no exchange of P^{32} between inorganic phosphate and virus. Also chemical analysis demonstrated that at least 99% of the P^{32} in the labelled virus was associated with the nucleic acid fraction. Within experimental error the specific radioactivity of the nucleic acid fraction was the same as that of the whole virus.

A small amount of the radioactivity of the virus was soluble in 5% trichloroacetic acid. The proportion varied from one preparation of virus to another up to about 1% but was usually less than 0.5%. It is considered, however, that this was radioactive impurity carried through the purification procedure rather than an integral part of the virus.

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ON THE ANTIHAEMOLYTIC VALUE OF THE BLOOD OF RABBITS AS MEASURED BY LYSOLECITHIN[1]

By H. B. Collier[2] and Helen L. Chen[3]

Abstract

The antihaemolytic values (AHV) of the erythrocytes and plasma of experimental rabbits have been determined by the lysolecithin method. Oral administration of cholesterol resulted in hypercholesterolaemia; and the increase in plasma AHV paralleled the rise in free cholesterol in approximately a 1 : 1 molar ratio of lysolecithin to cholesterol. No changes in the erythrocytes were observed, other than a mild anaemia. Splenectomy followed by cholesterol feeding afforded essentially the same results. In the normal rabbit it is concluded that the free cholesterol contributes very little to the antihaemolytic value of the plasma. Repeated bleeding caused a hypercholesterolaemia, and an elevation of the plasma AHV which paralleled the free cholesterol. In the haemolytic anaemia caused by acetylphenylhydrazine injections, the chief findings were an increase in hypotonic fragility of the erythrocytes, and a decrease in the plasma AHV. In all the experiments the mean erythrocyte AHV varied with the size of the cells, but appeared to be otherwise unaffected by the experimental procedures.

Introduction

Collier and Wilbur (10), in attempting to estimate the lysolecithin content of blood, described a method for measuring the amount of added lysolecithin (LL) which was required to produce 50% haemolysis of 1 ml. of blood under standard conditions. This amount, in mgm. of lysolecithin per ml. of blood, was termed the Antihaemolytic Value (AHV). It was assumed that there is normally an equilibrium between haemolytic and antihaemolytic factors in the circulating blood (8, 25) and that an alteration in this balance would be reflected in a change in the AHV. The chief haemolytic factors in the plasma are presumably fatty acids, bile salts, and lysolecithin; while the antihaemolytic substances are believed to be free cholesterol and the plasma proteins.

Other workers have attempted to estimate the antihaemolytic value of the blood by titration with lysins. Port (27) in 1910 used saponin for this purpose and found significant alterations in the serum of patients. Clark and Evans (5, 39) used saponin and also sodium oleate. Valette (35) measured the antihaemolytic value of serum to soaps, bile salts, and saponin; while de Vries (12) estimated the haemolytic activity of serum extracts against human erythrocytes.

May (21) in 1914 used saponin for determining the resistance of erythrocytes in disease. Singer (32) used lysolecithin for this purpose, and a haemolysis method for estimating the lysolecithin content of the serum of his patients. Foy and Kondi (16) and Maizels (20) also employed lysolecithin for determining the resistance of erythrocytes to haemolysis.

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Contribution from the Department of Biochemistry, University of Saskatchewan, Saskatoon, with financial assistance from the National Research Council of Canada.

[2] *Present address: Department of Biochemistry, University of Alberta, Edmonton, Alta.*

[3] *Present address: Department of Biochemistry, University of Michigan, Ann Arbor, Mich.*

In the present investigation lysolecithin was used for the measurement of the AHV of rabbit erythocytes* and plasma. The effects of splenectomy, of cholesterol feeding, and of acetylphenylhydrazine injection were observed.

Methods

All blood specimens were taken by heart puncture and heparinized, unless otherwise stated. Haemoglobin concentration was determined by the cyanmethaemoglobin method of Collier (6). The erythrocyte counts were performed upon dilutions in Gower's fluid; 10 squares in two fields were always counted, and the standard error of the count was found to be about 0.1 – 0.2 millions per μl. Packed erythrocyte volume ("haematocrit") was estimated in Wintrobe tubes centrifuged for 30 min. at 3000 r.p.m. in an International Clinical Centrifuge.

The free and ester cholesterol of plasma were separated by the digitonin procedure of Sobel and Mayer (33), and the estimations were made by the Rose, Schattner, and Exton (30) modification of the Tschugaeff reaction. As this affords a much more intense color than the Liebermann–Burchard reaction it is eminently suitable for estimating the low cholesterol levels of rabbit plasma.

A turbidimetric method for plasma protein, using sulphosalicylic acid, failed to give consistent results, and all plasma protein determinations were made by a micro-Kjeldahl procedure.

Blood AHV determinations were made by a modification of the method of Collier and Wilbur (10). In the original method the time for 50% haemolysis was specified as 60 sec. but it was subsequently found (7) that the action of the lysin required a much longer time to reach completion. For this reason certain preliminary measurements (9) are of little value. The AHV is now defined as: the number of mgm. of LL required for 50% haemolysis of 1 ml. of blood (or its equivalent) in 15 min. at room temperature.

The purity of the lysolecithin preparations was estimated by a determination of lipid phosphorus, and the figure of 6.08% P was taken as representative of what is actually a mixture of lysolecithin and lysocephalin.

The AHV of whole blood was determined as follows: Six small tubes were set up, each containing 5 ml. of 1 : 125 blood in buffered saline (10). Increasing amounts of LL (1 : 1000 in absolute ethanol) were added, the contents of the tubes were mixed, and after 15 min. the degree of haemolysis was estimated photometrically as previously described (10). The percentage haemolysis was plotted against the amount of LL added, and the amount required for 50% lysis was obtained by interpolation. The weight of LL, in mgm., is multiplied by 50 to obtain the AHV of 1 ml. of blood.

** It has been suggested that the term "Antihaemolytic Value" may not appropriately be applied to the erythrocytes. We agree with this contention, but retain the term in this sense, because of its use in previous publications. The AHV of erythrocytes could more properly be described as the 'resistance' to haemolysis by lysolecithin.*

The AHV of erythrocytes, after threefold washing in buffered saline, was determined in similar fashion, the results being expressed as mgm. of LL required for the erythrocytes from 1 ml. of whole blood. The mean corpuscular antihaemolytic value (MCAHV) was calculated by dividing the erythrocyte AHV by the number of cells per ml. of blood, and is expressed as $\mu\mu$gm. of LL per cell. The MCAHV represents, of course, only 50% of the mean LL required to lyse a single cell in the blood specimen.

The AHV of plasma was determined by adding a known volume of plasma to erythrocytes of known AHV and determining the AHV of the mixture. From the difference, the AHV of 1 ml. of plasma was calculated, and this represents essentially the amount of LL which can be neutralized by the plasma.

The hypotonic fragility, or osmotic resistance, of erythrocytes was estimated by the method of Parpart *et al.* (24). The blood was diluted 1 : 125 in buffered saline of varying tonicities, and the solutions were restored to isotonicity by addition of the complementary solutions before the opacity was read in the photometer. The results were then plotted on probability paper, as suggested by Bonét-Maury and Chouteau (3) and the fraction of isotonicity corresponding to 50% haemolysis was noted. This is designated as the Mean Corpuscular Fragility, MCF.

Rapoport (28) has pointed out the limitations of using only the MCF to designate the osmotic resistance; and both Bolton (2) and Ecker, Hiatt, and Barr (15) have recently described the advantages of plotting the first derivative of the percentage haemolysis curve, whereby any abnormal distribution in the osmotic resistance of the erythrocytes is made evident. In the present investigation it was observed that the fragility curves frequently did not give a linear plot on probability paper. In the distribution there was often a tendency to skewness toward the lower tonicities, but never any evidence of a bimodal distribution. For comparative purposes the hypotonic fragility was recorded simply as the MCF.

Results

Administration of Cholesterol

Cholesterol was administered orally in gelatine capsules. In the first experiment it was given at the rate of 1 gm. per day for 62 days to a rabbit weighing 3.1 kgm. The plasma became very opalescent and showed a high cholesterol content, and the results of the blood analyses are summarized in Table I. A slight anaemia was produced, but the most striking observation was the parallelism between the plasma free cholesterol and the plasma AHV, as illustrated in Fig. 1. The correlation coefficient between the free cholesterol and the AHV was $r = 0.93$; there was no correlation between the ester cholesterol and the AHV. In two similar experiments the rabbits survived only two weeks. In each case the plasma AHV rose as the free cholesterol increased.

TABLE I

THE EFFECT OF ORAL ADMINISTRATION OF CHOLESTEROL UPON THE AHV OF BLOOD OF A RABBIT*

(1 gm. of cholesterol daily for 62 days)

	Cholesterol feeding				No cholesterol			
	Time in days							
	0	7	22	44	66	75	90	95
Hb, gm./100 ml.	12.9	13.2	14.0	10.8	12.1	14.3	15.0	14.9
RBC, millions/μl.	5.71	6.00	6.10	4.60	4.78	5.57	6.32	6.20
Haematocrit, %	39.2	39.5	37.6	31.0	38.2	41.4	45.7	43.3
MCV, μ^3	69	66	62	67	80	74	72	70
Erythrocyte AHV, mgm./ml.	0.64	0.74	0.77	0.65	0.70	0.79	0.85	0.81
MCAHV, μμgm.	0.114	0.123	0.126	0.142	0.147	0.142	0.135	0.131
Plasma AHV, mgm./ml.	2.10	3.17	5.0	9.8	7.1	4.7	3.7	3.5
Plasma cholesterol								
Free, gm./100 ml.	0.019	0.084	0.152	0.464	0.284	0.227	0.102	0.062
Ester, gm./100 ml.	0.027	0.121	0.310	0.237	0.369	0.431	0.136	—

* *In all tables:* *MCV = mean corpuscular volume.*
MCAHV = mean corpuscular antihaemolytic value.
MCF = mean corpuscular fragility, fraction of isotonic.

These results appear to confirm the claim of Tsai and Lee (34) for the antihaemolytic potency of the plasma free cholesterol. Van Damme (36) has found that the antihaemolytic power of the plasma seemed to parallel the concentration of cholesterol and phospholipids. However, it is noted that when the curves in Fig. 1 are extrapolated back to zero value for free cholesterol the plasma AHV is still virtually at the normal level. It is concluded that *in the rabbit* the free cholesterol normally contributes very little to the plasma AHV.

The hypercholesterolaemia following cholesterol feeding has been described by Dubach and Hill (13) and by Popják (26). The former workers noted the production of a macrocytic anaemia; and Okey (22) and Okey and Greaves (23) observed a marked anaemia in guinea pigs fed cholesterol. In the present experiments only a mild anaemia was produced. No definite changes were observed in the erythrocytes of the cholesterol-fed rabbits, apart from the anaemia and the resulting slight macrocytosis.

Splenectomy and Cholesterol Administration

Kennedy and Okey (18) produced a severe anaemia in guinea pigs by administering cholesterol following splenectomy. In the present experiment, two rabbits were splenectomized and cholesterol feeding (0.6 gm. per day) was commenced one month after the operation. The results obtained with one rabbit are summarized in Table II. The splenectomy produced no apparent change in the plasma cholesterol or AHV, and the only observed

change in the erythrocytes was a decrease in hypotonic fragility. Administration of the cholesterol resulted in the usual hypercholesterolaemia and a rise in the plasma AHV, as represented graphically in Fig. 1. Only a moderate anaemia followed the cholesterol feeding.

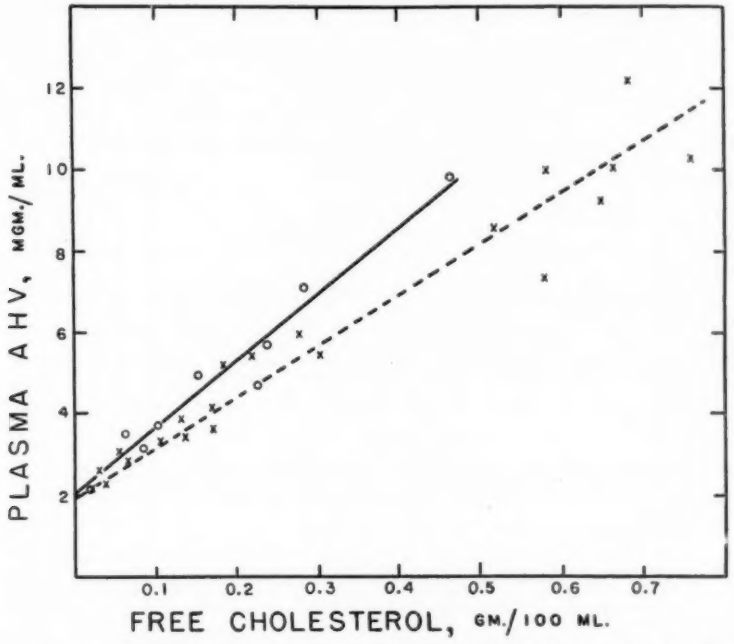

FIG. 1. *The relationship between plasma free cholesterol and plasma AHV. The straight lines were determined by the method of least squares and have the equations given below.*
O——O = *oral cholesterol.* $Y = 2.00 + 16.6\ X$.
X- - -X = *splenectomy followed by cholesterol.* $Y = 1.91 + 12.8\ X$.

The second rabbit died four weeks after the commencement of cholesterol administration but the results were similar to those noted above.

Effect of Repeated Bleeding

Two rabbits were bled repeatedly by ear vein. One animal, weighing 3.0 kgm., was bled a total of 226 ml. of blood over a period of 23 days, and the results of the analyses are recorded in Table III. A marked lipaemia resulted, and again the plasma AHV paralleled the free cholesterol level. The only significant change in the erythrocytes was a macrocytosis and an elevation of the MCAHV.

The second animal, weighing 1.9 kgm., was bled a total of 188 ml. over a period of 10 days. The erythrocyte picture was similar to that observed in the first rabbit, but there were no alterations in the plasma cholesterol or AHV.

TABLE II

THE EFFECT OF SPLENECTOMY FOLLOWED BY ORAL CHOLESTEROL UPON THE AHV OF BLOOD OF A RABBIT

(0.6 gm. cholesterol daily for 16 weeks, commencing one month after splenectomy)

	Splenectomy			Cholesterol administration				No cholesterol	
	Time, in weeks								
	0	1	3	5	10	16	20	24	27
Hb, gm./100 ml.	13.6	10.1	12.4	12.4	13.0	9.1	11.4	11.0	11.4
RBC, millions/μl.	5.54	4.64	5.41	6.30	6.16	3.60	4.72	4.88	5.84
Haematocrit, %	40.9	31.6	40.7	43.1	42.5	28.1	32.0	33.6	35.8
MCV, μ^3	74	68	75	68	69	78	68	69	61
MCF	0.516	0.520	0.455	0.463	0.547	0.475	0.563	0.516	0.525
Erythrocyte AHV, mgm./ml.	0.84	0.74	0.86	0.74	0.86	0.71	0.78	0.83	0.73
MCAHV, μμgm.	0.154	0.160	0.159	0.117	0.139	0.197	0.165	0.170	0.125
Plasma AHV, mgm./ml.	2.50	2.23	2.46	3.07	3.45	12.2	10.0	5.4	3.36
Plasma cholesterol									
Free, gm./100 ml.	0.025	0.033	0.023	0.053	0.132	0.68	0.58	0.217	0.106
Ester, gm./100 ml.	0.041	0.009	0.033	0.105	0.331	2.38	1.52	0.64	0.439

TABLE III

THE EFFECT OF REPEATED BLEEDING UPON THE AHV OF BLOOD OF A RABBIT

	Days									
	1	2	3	4	6	8	9	10	11	23
Volume bled, ml.	35	40	30	30	25	19	20	2	15	10
Hb, gm./100 ml.	12.2	10.2	7.4	6.0	5.9	6.8	7.3	7.2	6.8	13.3
RBC, millions/μl.	4.70	—	3.00	—	2.10	2.90	—	—	2.88	5.90
Haematocrit, %	36.0	—	22.6	—	19.8	24.4	—	—	24.0	41.0
MCV, μ^3	77	—	75	—	94	84	—	—	83	70
Erythrocyte AHV, mgm./ml.	0.65	—	0.39	—	0.33	0.39	—	—	0.35	0.64
MCAHV, μμgm.	0.138	—	0.130	—	0.155	0.135	—	—	0.120	0.109
Plasma AHV, mgm./ml.	1.83	—	1.69	—	2.34	3.07	—	—	2.19	1.87
Plasma free cholesterol, mgm./100 ml.	20	—	26	—	56	115	—	—	36	19
Plasma protein, gm./100 ml.	7.0	—	6.3	—	6.3	6.4	—	—	5.5	7.1

The hypercholesterolaemia following bleeding has already been described by the following workers: Horiuchi (17), Bloor (1), Chamberlain and Corlett (4), and Schwarz and Lichtenberg (31).

Acetylphenylhydrazine Anaemia

To investigate the blood changes in a typical haemolytic anaemia, acetylphenylhydrazine was injected intraperitoneally into four rabbits. The findings in two of the animals are represented in Fig. 2, and the results obtained with the other two animals were essentially similar.

The changes in the erythrocytes were those characteristic of a haemolytic anaemia: decrease in cell count and haemoglobin concentration, followed by an increase in the mean erythrocyte volume as immature cells were thrown

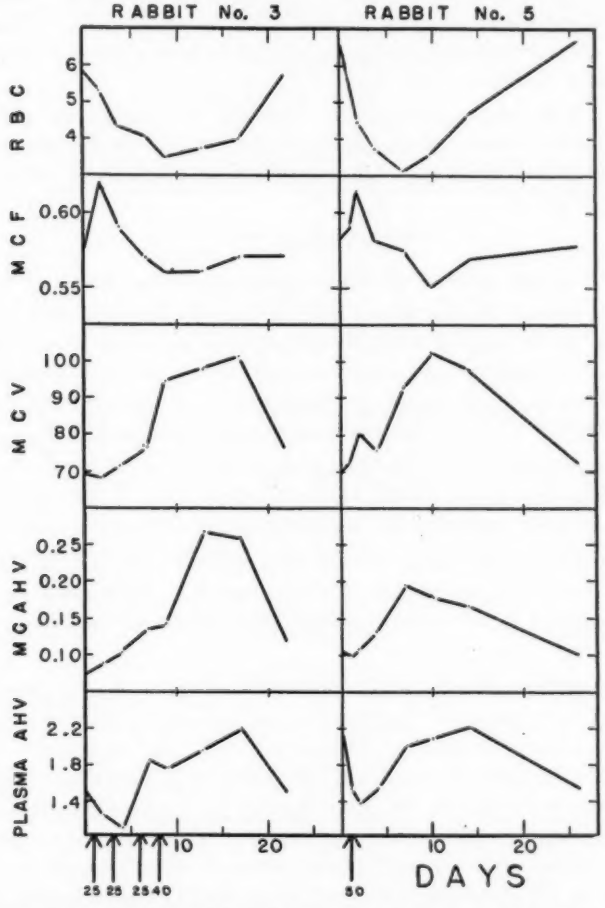

FIG. 2. *The effect of acetylphenylhydrazine injection upon rabbit blood. Arrows indicate injections of acetylphenylhydrazine, in mgm.*

RBC = erythrocyte count, in millions per μl.
MCF = mean corpuscular fragility, as a fraction of isotonic.
MCV = mean corpuscular volume, in μ^3.
MCAHV = mean corpuscular antihaemolytic value, in μμgm.
Plasma AHV = plasma antihaemolytic value, in mgm./ml.

into the circulation. This increase in cell volume in phenylhydrazine-induced anaemia has been described by Rapoport, Guest, and Wing (29) and by Lawrason and co-workers (19).

The drug had no apparent effect upon the mean AHV (MCAHV) of the erythrocytes, other than the increase which always accompanied an increase in the MCV. In both animals, however, there was observed a slight increase in hypotonic fragility which returned to normal in a few days. Numerous Heinz bodies were observed in the erythrocytes of all animals. These are characteristic of such drug-induced anaemias and have recently been discussed by Cruz (11) and by Webster (37, 38).

The plasma AHV decreased slightly following the treatment and then returned to normal. This may possibly be accounted for by the accelerating effect of acetylphenylhydrazine upon lysolecithin haemolysis which was previously reported (7).

Conclusions

1. The AHV of the plasma, in these experiments, was elevated by any treatment that caused an increase in plasma free cholesterol, such as oral administration of cholesterol and bleeding. It is probable that the free cholesterol neutralizes an equivalent amount of lysolecithin through the formation of a molecular complex; and experiments *in vitro* (7) have demonstrated the ability of added cholesterol to neutralize lysolecithin.

It may be noted that in Fig. 1 the molar ratios of lysolecithin to cholesterol, as represented by the slopes of the regression lines, are: 1.26 for the oral cholesterol experiment, and 0.97 for the splenectomy followed by cholesterol.

However, it must be emphasized that, although the observed *increases* in plasma AHV can be attributed to the increased free cholesterol, the free cholesterol of normal rabbit plasma apparently contributes very little to its AHV.

2. The mean antihaemolytic value (MCAHV) of the washed erythrocytes was remarkably independent of the experimental treatment, even in a haemolytic anaemia, where no decrease in the MCAHV was observed. These findings throw little light upon the mechanisms of drug anaemia, unless the observed decrease in the plasma AHV can account for increased destruction by lysins normally present. It may be noted that Dziemian (14) found an altered permeability of rabbit erythrocytes following phenylhydrazine injection, which may be related to our observation of an increase in the hypotonic fragility.

In our experiments the mean erythrocyte AHV appears to depend upon the size of the erythrocytes, and doubtless upon their composition. Dziemian (14) found that in phenylhydrazine-induced anaemia in rabbits the mean erythrocyte total lipid and free cholesterol increased as the MCV increased. It may be suggested that the mean corpuscular AHV is correlated with the cholesterol content of the erythrocytes. A rough calculation indicates that double the AHV of normal rabbit erythrocytes is of the same order of magnitude, on a molar basis, as the cholesterol content. However, changes in the erythrocyte cholesterol were not measured in our experiments.

Acknowledgments

We wish to acknowledge the kindness of Erica Lepp, of the Department of Physiology, in carrying out the splenectomies on the rabbits. Our thanks are due to Jean Pratt Milner for assistance in some of the early experiments.

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DEMONSTRATION OF AGGLUTININS FIVE HOURS AFTER INTRAPERITONEAL INJECTION OF PNEUMOCOCCUS TYPE I IN GUINEA PIGS[1]

By Doris S. Nunes[2]

Abstract

The inoculation of guinea pigs with pneumococci Type I intraperitoneally resulted in the development of homologous agglutinating antibodies, which were detected in the sera as early as five hours after inoculation. The early appearance of active immunity, and the attainment of a sufficient titer, would appear to govern survival to a fatal homologous re-infecting dose of the organism.

Introduction

Type specific antipneumococcus active immunity in experimental animals is recognizable by increased resistance to homologous infection (6), as well as by the usual test-tube titrations of agglutinins, precipitins, etc. This resistance to re-infection may be present before and may persist after circulating antibodies are detectable *in vitro* (3). Circulating antibodies have been demonstrated by agglutination and other techniques in varying periods from three to four days after the injection of antigen (3, 4), and many factors have been shown to influence the amount of antibody produced and the time of its appearance.

The purpose of this paper is to report on the appearance of homologous circulating antibodies, detected by agglutination tests, in 5 and 16 hr. after injection of the antigen and before therapy was instituted (15). According to the prevailing literature, this would appear to be the earliest demonstration on record (3, 17, 9, 11).

Materials and Methods

Test Organism

A virulent strain of *Diplococcus pneumoniae* Type I was used as the infecting organism throughout these experiments.*

Virulence for Guinea Pigs

Guinea pigs are usually less susceptible to pneumococci than are mice. The pneumococcus strain S–710–T1* showed a 100% mortality in mice in a 10^{-7} dilution of an 18-hr. culture, and a 50% mortality in a 10^{-8} dilution. Guinea pigs tolerated 1 ml. intraperitoneally of an 18-hr. culture without any clinical signs, and they recovered although bacteraemia was evident six hours after injection and even earlier in some of the animals. In order to ensure a fatal

[1] *Manuscript received in original form May 18, 1950, and as revised, September 1, 1950.*
From the Department of Bacteriology and Immunology, McGill University, Montreal, Canada.

[2] *Medical Research Fellow of the National Research Council of Canada.*

* *This strain, S-710–T I, was obtained from Dr. G. W. Rake, The Squibb Institute for Medical Research, New Brunswick, N.J.*

infection in guinea pigs, phagocytosis was blocked by simultaneous intraperitoneal injection of carmine and pneumococcus culture. Carmine, given either some hours before or at the time of injection of meningococci intraperitoneally, markedly raises the apparent virulence of the culture (1, 14). Therefore, 100 mgm. carmine in 1 ml. sterile saline was injected intraperitoneally into guinea pigs followed by 1 ml. of a 10^{-2} dilution of an 18-hr. culture of pneumococcus Type I containing approximately 12.5 million viable organisms per ml. which produced a 50% mortality rate in guinea pigs. Viability counts were done by "the drop method" described by Reed and Reed (18).

Animals

Groups of guinea pigs, all weighing between 500–600 gm. were chosen. These were kept under observation for at least a week prior to infection, and a preliminary bleeding taken in order to determine that no specific circulating antibodies for the test organism existed.

Agglutination Tests

Halving serial dilutions of each serum in 0.2 ml. volume in 0.85% saline ranging from a 1 in 2 dilution to a 1 in 2048 dilution were made in clean dry Kahn tubes; the control containing 0.2 ml. saline only. An equal volume of standard antigen was added to all tubes, which were placed in a water bath at 37° C. for two hours and then in the refrigerator overnight at 4° C. The tubes were read for agglutination against a viewing-box having a black background with oblique light.

Preparation of the Antigen

About 100 ml. of "L.S."* broth was inoculated heavily with lyophilized S–710–TI pneumococci cultures and incubated at 37° C. for 18 hr. The cultures were killed by heating for one hour at 60° C. and centrifuged. The organisms were washed four times with 0.85% saline, and finally diluted with saline to a concentration of No. 2 McFarland nephelometer and stored in the refrigerator at 4° C. The antigen was finally tested for Gram-positiveness, capsule swelling, and for sterility by aerobic and anaerobic cultures. In addition, no antigen more than a week old was ever used.

Variation in Consecutive Antigen Preparations

Several lots of antigen had to be prepared during a series of experiments, and many showed variation in agglutinability when prepared by the same or different procedures. Variation in agglutinability could not be directly related to size of tubes, temperature, pH, medium, time, shaking on addition of antigen, and virulence of the organisms, all of which were checked repeatedly. Instead of compensating for this, by assigning to each preparation an agglutinability factor, which might be difficult in weak sera, a standard immune serum was used, to select antigens of suitably sensitive agglutinability (See Fig. 1).

* *Special culture media used in the Department of Bacteriology, McGill University.*

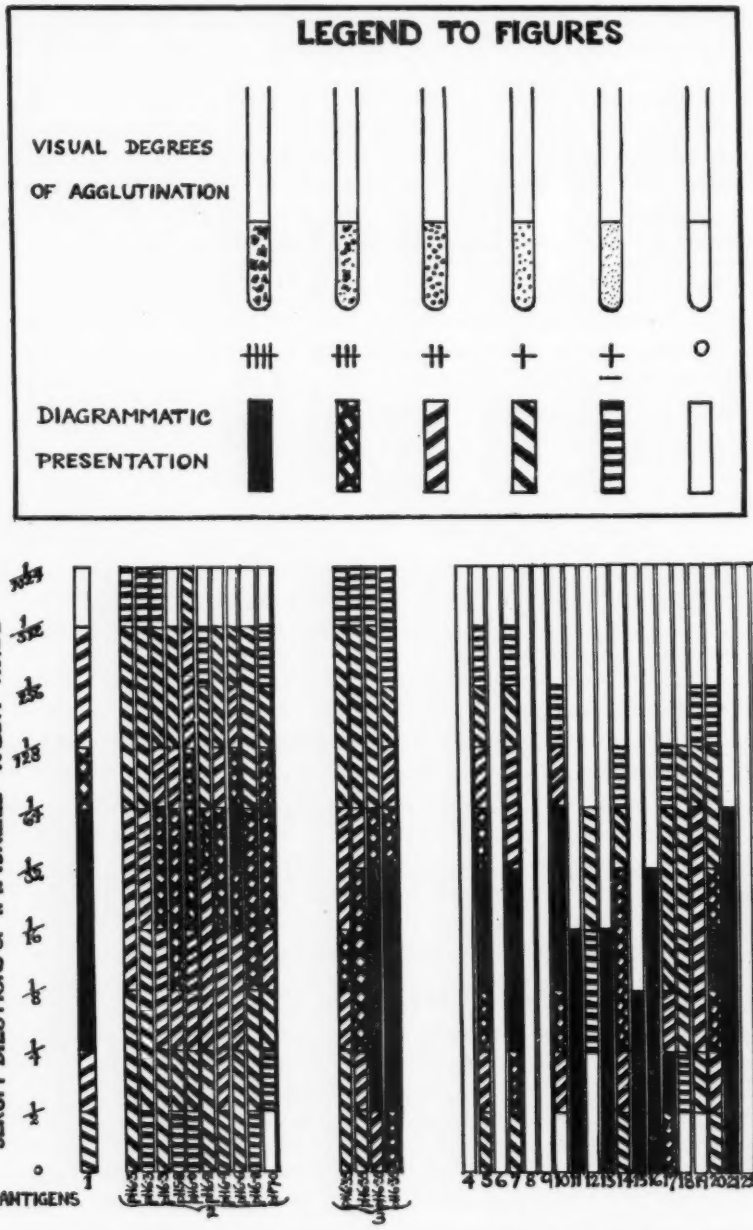

FIG. 1. *Variation of agglutinability of pneumococci Type I antigens, using the same immunized Type I pneumococcic rabbit serum.*

Experimental

Agglutinating Antibodies Five Hours after Intraperitoneal Injection of Pneumococcus Type I

Procedure

Selected guinea pigs (No. S1–S18 inclusive) were bled by cardiac puncture five hours after the standardized intraperitoneal injection of carmine and pneumococcus Type I culture. In five hours, the animals all showed clinical signs of infection as seen by ruffled fur, lethargy, and apathy to stimulation. The infection was then controlled by sulphamethazine immediately after this bleeding and for the next four to five days.

Another group of guinea pigs (No. P1, P3–P26 inclusive) were similarly injected intraperitoneally, then bled in five hours. Penicillin was the therapeutic agent used in this group.

Subsequent bleedings were taken in 5, 10, and 20 days after infection in both groups of animals, and in addition, cutaneous tests to Type I capsular polysaccharide,* and homologous re-infection with the original infecting dose were made on the 20th day.

Cutaneous tests were made by the intracutaneous injection of 0.1 ml. containing 0.0004 mgm. of Type I capsular polysaccharide on the shaved abdominal wall. A positive reaction was taken as an area of erythema surrounding the site of injection, usually appearing within a half hour, with absence of any reaction in the control which consisted of saline only.

A third group of guinea pigs (No. I1–I5 inclusive) were injected in the same way, then immediately afterwards given their first dose of sulphamethazine. These animals were bled in five hours, again in 16 hr., in five days, 10 and 20 days. These were similarly subjected to cutaneous tests with Type I polysaccharide and re-infection on the 20th day.

Results

In the S series, seven out of the 18 sera showed agglutinating antibodies varying from a 1 in 32 dilution to a 1 in 256 dilution. These were not very high in amount, as + agglutination was the highest recorded, and all except one serum showed prezoning. Eleven of the sera were negative in all dilutions.

In the P series, there were demonstrable agglutinating antibodies in five out of 25 sera, varying from a 1 in 4 dilution to a 1 in 64 dilution, with ++ agglutination the highest recording, and only two sera showed prezoning. Twenty sera were negative in all dilutions (Fig. 2).

In the I series, none of the sera exhibited agglutination.

In addition, it was noted that the antibody titer achieved by the 20th day in these series of animals appeared to have some definite bearing on the response of the animals to re-infection (Table I) viz.: guinea pigs S3, S6,

* *Obtained from the Squibb Institute for Medical Research, New Brunswick, N.J.*

TABLE I

COMPARISON OF CUTANEOUS TESTS AND RE-INFECTION TO THE ANTIBODY TITER ON THE 20TH DAY

Guinea pig No.	Antibody titer (five hours)	Antibody titer (20th day)	Cutaneous test to Type I capsular polysaccharide (20th day)	Survival to re-infection (20th day)
S 3	1 : 32	(Died)	0	0
S 6	1 : 128	(Died)	0	0
S 8	1 : 128	1 : 32	+	+
S 15	1 : 128	1 : 1024	+	+
S 16	1 : 256	1 : 512	+	+
S 17	1 : 32	1 : 512	+	+
S 18	1 : 32	1 : 512	+	+
P 8	1 : 8	(Died)	0	0
P 9	1 : 4	(Died)	0	0
P 11	1 : 4	(Died)	0	0
P 18	1 : 64	1 : 512	+	+
P 20	1 : 16	1 : 512	+	+
I 1	0	1 : 64	+	+
I 2	0	1 : 128	+	+
I 3	0	(Died)	0	0
I 4	0	1 : 256	+	+
I 5	0	1 : 256	+	+

P8, P9, P11, I3. These six animals demonstrated titers varying from 0 to 1 in 128 dilution in five hours, however, did not survive until the 20th day. Those that did survive demonstrated titers of 1 in 64 dilution or higher by the 20th day, with the exception of guinea pig S8, and were able to resist re-infection.

AGGLUTINATING ANTIBODIES 16 HR. AFTER INTRAPERITONEAL INJECTION OF PNEUMOCOCCUS TYPE I

Procedure

Guinea pigs (Nos. S′1–S′20 inclusive) were bled 16 hr. after the standardized intraperitoneal injection of carmine and pneumococcus Type I culture. Clinical signs of infection were more marked than in five hours, as may be expected. Sulphamethazine was then administered as in the S series.

Guinea pigs (Nos. P′2–P′15 inclusive) were similarly injected intraperitoneally, then bled in 16 hr. after which the first dose of penicillin was given as in the P series.

Both these groups, S′ and P′, were similarly bled in 5, 10, and 20 days after injection, and tested cutaneously with Type I capsular polysaccharide and homologous re-infection on the 20th day.

A third group of guinea pigs (Nos. I1–I5 inclusive) were injected in the same way, then immediately afterwards given their first dose of sulphamethazine. These animals were bled in five hours, again in 16 hr., in five

days, 10 and 20 days. These were similarly subjected to cutaneous tests with Type I polysaccharide and re-infection on the 20th day.

Results

In the S′ series, four out of 20 animals showed agglutinating antibodies varying from a 1 in 8 dilution to a 1 in 64 dilution with prezoning in one serum only, and ++ agglutination was the highest reading. Sixteen sera were negative in all dilutions.

In the P′ series, agglutinating antibodies could be demonstrated in 6 out of 14 sera. The titers in this group were the highest obtained, varying from a 1 in 8 dilution to a 1 in 2048 dilution, with ++ agglutination the highest recording, and three out of six sera exhibiting prezoning. Eight sera were negative in all dilutions (See Fig. 2).

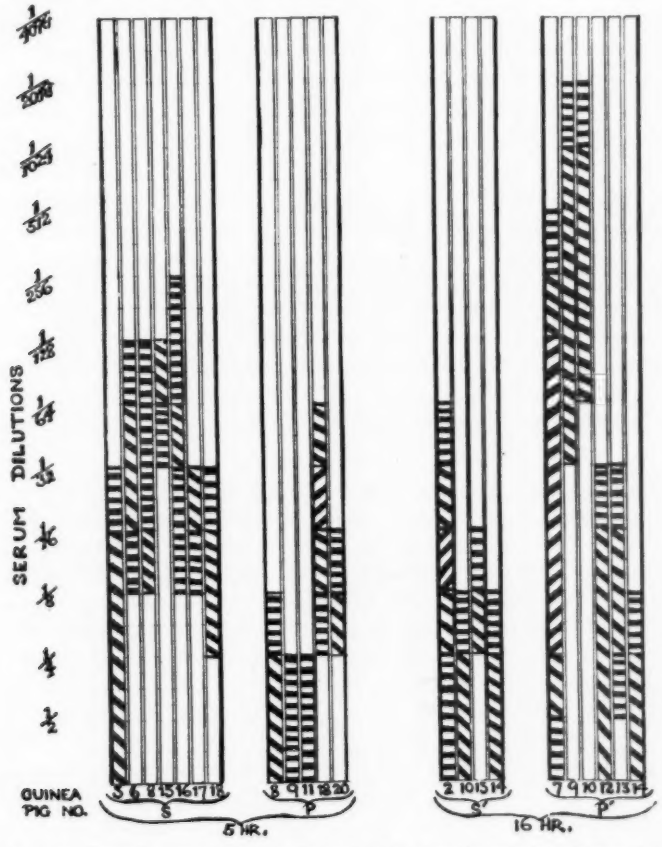

FIG. 2. *Agglutination titers in five hours and 16 hr. after inoculation of Type I pneumococci intraperitoneally in guinea pigs.*

In the I series, none of the sera exhibited agglutination.

As in the previous series, the titer at time of re-infection bore a definite relationship to survival and therefore some prediction of the outcome could be made (See Table II).

TABLE II

COMPARISON OF CUTANEOUS TESTS AND RE-INFECTION TO THE ANTIBODY TITER ON THE 20TH DAY

Guinea pig No.	Antibody titer (16 hr.)	Antibody titer (20th day)	Cutaneous Test to Type I capsular polysaccharide (20th day)	Survival to re-infection (20th day)
S' 2	1 : 64	(Died)	O	O
S' 10	1 : 8	1 : 1024	+	+
S' 13	1 : 16	1 : 1024	+	+
S' 14	1 : 8	1 : 128	+	+
P' 7	1 : 512	1 : 128	+	+
P' 9	1 : 2048	1 : 128	+	+
P' 10	1 : 2048	1 : 64	+	+
P' 12	1 : 32	1 : 8	+	O
P' 13	1 : 32	(Died)	O	O
P' 14	1 : 8	1 : 128	+	+
I 1	0	0 : 64	+	+
I 2	0	1 : 128	+	+
I 3	0	(Died)	O	O
I 4	0	1 : 256	+	+
I 5	0	1 : 256	+	+

In these groups, only guinea pig P'12 had a titer of 1 in 8 dilution by the 20th day, and failed to survive re-infection; those surviving demonstrated a titer of 1 in 64 dilution or higher by the 20th day.

In addition to the titer finally achieved by the 20th day, there appeared to be a further relationship between the *early* formation of antibodies and a greater resistance to re-infection (See Table III).

TABLE III

EARLY APPEARANCE OF ANTIBODIES AND RESISTANCE TO RE-INFECTION IN GUINEA PIGS

	5-Hr. group		16-Hr. group	
	Antibody present	No antibody present	Antibody present	No antibody present
Total No. of guinea pigs	12	36	10	29
No. guinea pigs dying before 20th day	5	20	2	16
No. guinea pigs surviving to 20th day	7	16	8	13
No. guinea pigs resistant to re-infection on 20th day	7	11	7	11

Discussion

The higher survival rate of the guinea pigs which formed antibodies in less than 16 hr. indicated a greater resistance to re-infection than those animals which were tardy in producing antibodies. In the latter group, only 29 out of 65 survived until the 20th day, and of these 22 only resisted re-infection. This was in contrast to those early antibody producers, of which 15 out of 22 survived until the 20th day, and then only one succumbed to re-infection.

In guinea pigs (11–5 inclusive) which received sulphonamides immediately after infection, the first appearance of antibodies was late (10th day), and these animals appeared resistant to re-infection on the 20th day. In view of the small group of animals used here, it is difficult to draw any conclusions as to their real or apparent resistance.

The toxicity of penicillin for guinea pigs as a species (8, 16) was a definite handicap, and produced per se a higher mortality in the penicillin treated animals. However, this factor was present for both the early and late antibody formers in the P and P′ series. It was also noted that five out of 39 of the guinea pigs treated with penicillin demonstrated grossly fatty sera, in which estimations for fatty acid gave results ranging from 30 to 285 milk equivalents (13).

It would therefore appear that the early evidence of an immune response, as demonstrated by circulating antibodies, would predict a lasting and effective state of immunity, and resistance to re-infection. This was shown in the lower mortality rate of this group when re-infected with the original re-infecting dose, as compared to those in which antibodies were later in appearing. In addition to this, the titer at time of re-infection was an important criterion even in those animals which produced antibodies within 16 hr., and, for the standard antigen used, a maximum titer of a 1 in 8 dilution was insufficient to ensure protection. A safe minimum was found to be agglutination in a 1 in 64 dilution.

No definite explanation for the remarkably early appearance of circulating antibodies can be offered. The reticuloendothelial system (9, 10) and lymphocytes (2, 7) have been credited with the production of antibodies in the body. Hoder (9) has shown that damage to the reticuloendothelial system resulted in a decrease in antibody production, and stimulation in an increase. Whereas carmine was used effectively in raising the apparent virulence of the organism, it may also play some role in maintaining antibody formation. Several unrelated adjuvants have been used to sustain antibody formation (5), by probably protecting the antigen against destruction and elimination. However to attribute carmine with the property of increasing the rate of antibody production, further investigation would be necessary. Carmine does not appear to affect the apparent health of mice. It is first "agglutinated" into masses, phagocytosed, and eventually removed by the peritoneum and omentum. The response of the animals to such an early production of antibodies appeared almost in the nature of an allergic or hypersensitive reaction.

Preliminary bleedings, however, ruled out any possibility of previous sensitization to the test organism, and no adverse clinical after-effects were noted.

Löfström (12) has reported a nonspecific capsular swelling substance of pneumococcus formed in the early stages of bacterial disease, and associated with the α and β globulin fractions of serum proteins. This substance is produced after the subcutaneous injection of pneumococci Types I and III in rabbits. It appears within 24 hr., attaining a maximum in two days, disappears in a week, and agglutinates pneumococci Types XVI, XXVII, and XXVIII. However, this substance has not been demonstrated in horses or guinea pigs.

Assuming that favorable conditions existed in the choice of the antigen, and the route chosen permitted a large absorptive area, the agglutinating titers obtained in 5 and 16 hr. were enormous. Furthermore, the variability of the antigen was controlled, so no untoward sensitivity can be blamed.

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